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# Exposure of Neonatal Porcine Islet Cells to Human Cytokines Reduces Cellular Insulin Content but not Viability

by

**Jeffrey A. Toreson** ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Surgery

Department of Surgery

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**University of Alberta**

**Faculty of Graduate Studies and Research**

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Exposure of Neonatal Porcine Islets to Human Cytokines Reduces Cellular Insulin Content but not Viability** submitted by **Jeffrey A. Toreson** in partial fulfillment of the requirements for the degree of **Master of Science** in **Experimental Surgery**.

*This text is dedicated to my family, who have given me everything I need to succeed,*
*Thank you for your never ending support*

# *Abstract*

***Background***-Limited donor tissue for islet transplantation has led to a search for alternate islet sources. Porcine neonatal islet cells (NIC) are an attractive source because of their inherent ability to differentiate and proliferate. Human cytokines, IL-1β, IFNγ and TNFα, may affect neonatal porcine islets.

***Methods***- Porcine NIC were exposed to IL-1β, IFNγ and TNFα for 48 hours in vitro. They were compared to NIC with no exposure to cytokines for insulin/DNA recovery, secretory response, PDX-1 protein and porcine insulin mRNA expression and, caspase 3 activity.

***Results***- NIC insulin secretion was not effected by human cytokines (2.63± 0.29-exposed vs. 2.71± 0.22 –control), however, total cellular insulin was reduced in exposed islets versus controls (56.6% ±8.13 vs. 104.4 ±12.51 respectively, $p<0.01$). There was no significant difference in the DNA recovery, caspase 3 activity and PDX-1 or insulin mRNA expression.

***Conclusions***- Human cytokines do not effect the secretory response of neonatal porcine islet cells, nor are they cytotoxic in vitro. These cytokines do lower the total cellular insulin of neonatal porcine islets.

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# List of Abbreviations

| | |
|---|---|
| NIC | neonatal islet cell |
| IDDM | insulin dependent diabetes mellitus |
| MHC | major histocompatibility complex |
| GAD | glutamic acid carboxylase |
| GABA | gamma-aminobutyric acid |
| NOD | non-obese diabetic |
| Scid | severely compromised immuno-defficient |
| TCR | T-cell receptor |
| IL-1β | interleukin one beta |
| TNFα | tumor necrosis factor alpha |
| IFNγ | interferon gamma |
| Th | T- helper |
| PDX-1 | pancreatic duodenal homeobox one |
| PC ½ | proinsulin convertase one/two |
| NOS | nitric oxide synthase |
| ATP | adenosine triphosphate |
| IRE | interferon response element |
| IRAP-1 | interleukin receptor agonist protein |
| NMMA | $N^G$-monomethyl-L-arginine |
| NAME | N-ω-nitro-L-arginine methyl ester |
| cGMP | cyclic guanosine monophosphate |
| DNA | deoxyribonucleic acid |
| mRNA | messenger ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| ICE | interleukin converting enzyme |
| LPS | lipopolysaccharide |
| HSP | heat shock protein |
| APC | antigen presenting cell |
| NPIC | neonatal porcine islet cell |

| | |
|---|---|
| SLA | swine leukocyte antigen |
| PBL | peripheral blood leukocyte |
| CTL | cytotoxic lymphocyte |

# Introductory Chapter

Insulin dependent diabetes mellitus, or IDDM, is a disease resulting from an autoimmune assault on insulin producing beta cells. These cells are located in the islets of Langerhans in the pancreas. The loss of beta cell mass results in an inability to produce insulin, a crucial hormone in the regulation of glucose levels. The result is abnormal glucose metabolism yielding chronic hyperglycemia.

Diabetes is not a disease that occurs rapidly. In fact, IDDM is a progressive disease in which beta cells are gradually destroyed over a number of years. Eisenbarth separates the development of diabetes into five stages (1). Stage one is referred to as genetic susceptibility. Diabetes seems to be a polygenic disease in which certain major histocompatibility complex (MHC) and non- MHC alleles may increase ones chance of developing the disease (2). Stage two involves the "triggering" or inception of the disease. This event is often thought to be an environmental factor such as a viral infection (1,3,4). The third stage is that of active immunity where we detect the first signs of an autoimmune response towards the islet derived beta cells. This progresses into the fourth stage of diabetes in which we see a loss of normal glucose- stimulated insulin secretion. Often in this stage there is a poor first phase glucose response during a hyperglycemic challenge. The final stage in the disease progression is the development of overt diabetes. In this period individuals present with severe chronic hyperglycemia; already, most of the beta cell mass has been destroyed. Since diabetes is not usually diagnosed until the overt stages of the disease this presents a serious dilemma. At this time, the majority of the beta cells have already been destroyed, therefore, prevention is almost impossible at the first stages of hyperglycemia. It is very clear that new methods of disease diagnosis that are able to predict the disease in earlier stages are required for prevention or immune therapies to work.

The abnormal glucose metabolism and chronic hyperglycemia of diabetic patients poses a serious threat. The development of insulin therapy has drastically improved the quality of life of inflicted individuals by allowing them to manage their blood glucose levels. Insulin, therapy, however, does not prevent transient periods of

hyperglycemia. These short episodes can cause many serious complications such as retinopathy, neuropathy, nephropathy and cardiovascular disease. Diabetics also have an increased susceptibility to certain infections such as psuedomonas, or monilial skin infections. Periarticular thickening of the skin may lead to decreased motility of the fingers. Most likely the causes of these complications are due to the indirect and direct effects of hyperglycemia. The Diabetes Control and Complications Trial demonstrated that long-term intensive insulin treatment was associated with a reduction in the risk of developing diabetes-related complications (5). Hyperglycemia most likely mediates its damage in several ways; increased glycosylation of protein and hemoglobin, inducing abnormalities in vascular endothelium, and supporting pericytes in the retina and mesangial cells in the glomerulus. Last, the duration of diabetes and degree of hypertension seem to be risk factors for the development of these complications (6).

## Immunology of type 1 diabetes

Insulin dependent diabetes mellitus is an autoimmune disease in which islet beta cells are selectively destroyed. Susceptibility to diabetes depends on a number of genetic and environmental factors. It is important to understand the immunology of diabetes since it plays an important role in disease pathogenesis, autoimmune rejection and allograft rejection. Several areas of importance are: genetics (role of the major histocompatibility), possible islet cell autoantigens, triggering (role of environmental factors), insulitis, T-cells and cytokines.

Clearly, diabetes is a heterogeneous disease in which there are many genetic factors influencing its inception. However, most authors believe that the genotype of an individuals major histocompatibility complex seems to be the best determinant for possible susceptibility to diabetes (3). For example, polymorphisms in the human leukocyte antigen class two MHC alleles seem to correlate directly with disease susceptibility or protection (2,7) . This is logical since class two MHC receptors are responsible for binding foreign, extracellular antigenic peptide and presenting them to CD 4+ T cells. This interaction is involved in activating T helper cells in order to

develop a response to infection. Class two MHC may be directly involved in the inception of diabetes by binding peptides that evoke an anti- islet T cell response. Class two MHC is a heterodimeric glycoprotein consisting of an alpha and beta sub-unit (8). The three class two MHC loci in humans are the DR, DP, and DQ loci. Most literature states that the two most susceptible alleles are HLA A1, B3 DR3 DQB1 0210, and HLA DR4 DQB1 0302 (2,3,7). The amino acid at position 57 in these two alleles seems to be very important. Normally position 57 is an aspartic acid, however in these two alleles there is usually a substitution of a small aliphatic residue. This allows a larger array of peptides with negative charges to bind. One theory states that the amino acid sequence change alters the type/ amount of peptides presented by class two MHC subset. This may make the presentation of diabetogenic/ self-antigen more likely in people carrying these susceptible alleles (2,3,7). Interestingly, certain MHC-class two haplotypes confer protection against diabetes. This resistance is noticed even if the protective haplotype is combined with a MHC haplotype conferring susceptibility. In a review, Noorchasm et al state that the protection may be due to competition (2). Protective class two MHC may provide resistance by out competing susceptible MHC for diabetogenic peptides. This competition may result in difficulty turning on autoimmune T cells. Bach, in his review, suggests another mechanism for defense. Protective MHC class two alleles may be capturing auto-antigen and presenting them to T-suppressor cells of the Th2 variety. The engagement of these suppressor cells may shut down an anti-beta cell response (3).

More than one peptide/MHC complex may be pathogenic. By analyzing peptide-binding motifs, we are able to infer something about the nature of diabetogenic antigens. Noorchasm warns that some peptides which bind class two MHC are indiscriminant in that they bind many MHC molecules regardless of their binding motif. If, in fact, diabetogenic peptides are indiscriminant the study of MHC class two molecules may be irrelevant (2).

Since Class two MHC seem to be intimately involved in the pathogenesis of diabetes, it is crucial to discover the particular auto- or diabetogenic antigens that may be recognized by these receptors in order to elicit an anti beta cell response. Tisch, in

his review lists two important criteria for autoantigens: they must be correlated with the disease in man or animal models, and treatment with autoantigens must modulate diabetogenic response in NOD mice. There does not seem to be one precise autoantigen, several may be playing a role in the inception of IDDM.

One possible autoantigen is glutamic acid decarboxylase, or GAD for short. This enzyme exists in two isoforms- GAD 65 and GAD 67. GAD plays a role in the biosynthesis of the neurotransmitter gamma-aminobutyric acid, or GABA for short. GAD is secreted in both the brain and beta cells. The presence of anti-GAD antibodies seems to be a predictive marker for diabetes (9). The presence of anti-GAD antibodies has also been observed in NOD mice (7). The recognition of anti-GAD occurs early on in the diabetic process and it may be involved in mediating the initial events associated with insulitis. Kaufman et al realized that immunization of young NOD mice with GAD helped to prevent the later occurrence of insulitis and diabetes (10).

We also see that there is a correlation between the appearance of anti-insulin antibodies and the onset of diabetes. In fact, fifty percent of diabetics with recent occurrence of the disease test positive for anti-insulin antibodies (3). Interestingly, insulin reactive T cells can accelerate the disease in NOD mice, and can adoptively transfer the disease in NOD-Scid mice (11).

What is it that triggers the onset of diabetes? Environmental factors such as viral infection may propel an otherwise normal individual into an autoimmune response against beta cells. Most of the literature on the immunology of diabetes is in agreeance that environmental factors such as infection play a role in diabetes inception (1,3,4,7). In his review, Bach provides many insights into the role of virus in diabetes. Certain viral strains such as encephalomyocarditis virus in mice, and rubella in hamsters play a role in diabetes development. This provided greater impetus for the study of viral-induced diabetes (3). Viruses most likely help cause diabetes in one of two ways; by either modulating the expression of pre-disposing genes or resistance genes that may effect disease development or by triggering a potent anti-islet immune response. Certain viral infections correlate to diabetic resistance in animal models. These viruses may be causing the expression of

protective genes, or inducing the display of viral or self-peptide that evoke T-suppressor cells.

In developing diabetes, a condition known as insulitis develops. This condition occurs with the accumulation of macrophages, T cells, dendritic cells and eosinophils in pancreatic tissue. The first stage of insulitis is peripheral insulitis (3). Also referred to as peri-insulitis, it involves the build up of the former cells in the ductal spaces surrounding islet cells. As diabetes progresses, a condition called intra-insulitis develops in which invading mononuclear cells actually penetrate the islets. Macrophages are the first cell type to invade the islet bodies and may play and important role in IDDM through cytokine production (12). The dominating cell phenotype is CD4+ and CD8+ cells. Insulitis may cause the increased expression of adhesion molecules such as L-selectin and VLA-4 on leukocytes (3). This may cause the recruitment of leukocytes to the islets perpetuating insulitis. During insulitis, high levels of interferon gamma are noticed, which may be directly involved in the recruitment and activation of macrophages. It is unclear in the literature what degree of beta cell damage is done during insulitis. Whether beta cells are destroyed immediately during insulitis or is there a lag period in which islet function is merely inhibited is unknown. If the latter were the case, this would leave a window of opportunity between infiltration and beta cell destruction for immune therapy to correct and prevent diabetes occurrence.

T cells may be the main aggressors of beta cell loss of IDDM (1,3,7). This seems likely since adoptive transfer experiments in NOD mice show T cell preparations from diabetic animals can transfer the disease to non-diabetic animals (3,7). Anti- TCR antibodies injected into young NOD mice can also prevent disease occurrence (7). T cells, especially CD4+ cells, may be playing a role by producing cytokines and other immune mediating molecules that recruit and activate other leukocytes such as macrophages and eosinophils. Cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and interferon gamma (IFNγ) may increase MHC expression, inhibit islet function and cause changes in beta cell phenotype (reviewed later in article).

Since T cells may directly cause islet cell destruction, it is important to understand mechanisms in which T cells may lose tolerance. Tolerance is the lack of an immune response towards self-antigen. In diabetes, we see a clear loss of tolerance for insulin positive cells. Tolerance is created through several mechanisms such as negative selection in the thymus, anergy and the presence of T suppressor cells (8).

## Role of cytokines in type 1 diabetes

Cytokines are pleiotropic messengers used by the immune system to communicate, control local or systemic responses and produce inflammation (8). Cytokines are generally secreted by T cells, macrophages and some other leukocytes. There are over thirty different types of cytokines (4) and they are important effector molecules in the pathogenesis of diabetes. It is thought that poor regulation and imbalanced cytokine expression may result in a potent anti- beta cell response (4,13).

T helper cells, by producing various cytokines, are able to regulate how a host responds to a pathogenic challenge. There are two main subsets of T helper cells: Th1 and Th2 responses. T helper cells are not pre-committed to either subset. In fact, it is thought that the cytokine environment that is present as antigen-primed T helper cells differentiate determines the subset that develops(8). Two cytokines in particular are important in determining whether a Th1 or a Th2 response develops. IL-4 encourages a Th2 response while IL-12 favours the development of a Th1 response. Th1 cells produce IL-2, IFN$\gamma$, TNF$\alpha/\beta$, all of which control cellular mediated immunity. A Th1 response, or pro-inflammatory response, causes changes in the architecture of the vascular endothelium resulting in the recruitment of various circulating leukocytes into the area of antigenic challenge (4,13). Macrophages are activated and in turn they produce IL-1$\beta$ and help in the phagocytosis of infected or altered cells. Moreover, IL-2 production helps activate cytotoxic T-lymphocytes that are able to kill cells displaying antigenic peptides. Th2 helper cells produce an entirely different array of cytokines. These include IL-4, IL-5, and IL-10, which stimulate a potent humoral response involving B cells and immunoglobulin. Th1

responses are best suited for viral infection while humoral responses are well suited for allergens and gut parasites (13).

Interestingly, these two responses also appear to be able to cross talk and regulate one another. If a Th1 response is mounted, interferon gamma feeds back and inhibits the humoral immunity by shutting down Th2 cells. Likewise, if a Th2 response is initiated, IL-4 and IL-10 can prevent a Th1 response from occurring (8). This is very important since it allows the host to exclusively choose the response best suited for an antigenic challenge.

In diabetes there is evidence that a Th1 response is pathogenic (4,13). Pro-inflammatory responses promote leukocyte infiltration into the beta cell mass. This allows for the specific delivery of cytokines such as IL-1β, TNFα and IFNγ to the insulin positive tissue. Th2 responses, moreover, seem to be protective against diabetes. In his review Rabinovitch states that IL-4, IL-4 secreting T cells and IL-10 may all prevent the onset of IDDM (4)

## Pro-inflammatory cytokines and their effects on islets

Th1 responses producing IL-1β, IFNγ, and TNFα can be cytotoxic to islet cells. The first evidence that cytokines may play a crucial role in the pathogenesis of IDDM was discovered by Thomas Mandrup Poulsen et al in 1986 (14). It should be noted that most of the early studies on cytokine induced effects on islets were done using rat islets. Poulsen suspected that during insulitis leukocytes might play a role in inhibiting islet function and stimulating islet degeneration by secreting various cytokines (both monokines and lymphokines). In his studies, supernatant was isolated from blood mononuclear cells stimulated with lectin. This supernatant was shown to markedly inhibit insulin release from both human and rat islets, more so in rat islets. Moreover, electron microscopy showed degeneration of islets exposed to the supernatant. Studies on the supernatant showed that it was most likely a protein substance causing these effects. Poulsen speculated that a cytokine would be found responsible.

Since activated mononuclear cells secrete a variety of biologically active molecules, it was necessary to isolate the specific “cytokine” causing the effect seen above. In 1987, Poulsen et al used affinity chromatography to purify IL-1β, IL-2, macrophage inhibition factor and other molecules from the supernatant of activated mononuclear cells (15). In their study, they showed that it was IL-1 that was responsible for the inhibition and degeneration of rat islets. This was the first study to show that IL-1 could affect insulin release either by affecting insulin biosynthesis or by causing beta cell death.

In 1987, Sandler et al cultured rat islets for forty-eight hours with IL-1β, IFNγ, and TNFα alone. IFNγ and TNFα alone failed to effect rat islet function or morphology (16). IL-1β was able to significantly decrease insulin secretion in basal and high glucose media. DNA content after culture in the presence of IL-1β was also significantly reduced. Proinsulin synthesis decreased from thirty percent to ten percent of total protein produced. This study failed to pinpoint a mechanism for IL-1β activity on rat islets but it was the first to show DNA damage and a reduction in proinsulin synthesis.

Finally, in 1990 a study by Southern and colleagues was able to suggest a possible mechanism for the action of IL-1β (17). In this study, rat islets were cultured in IL-1β, both with and without TNFα. IL-1β almost completely inhibited insulin secretion, and TNFα potentiated this effect. When the same in vitro experiment was done in L-arginine free media, the effects of IL-1β were reduced slightly. When this experiment was repeated with N-ω-nitro-L-arginine methyl ester, (NAME) an inhibitor of nitric oxide production, the effects of IL-1β and TNFα were almost completely relieved. This study was the first to implicate nitric oxide synthase, NOS, as a possible mediator. NOS converts L-arginine to nitric oxide and citrulline (18). Nitric oxide is thought to inactivate certain mitochondrial enzymes such as aconitase impairing cellular respiration and function (4,12,17,19). No other signal cytokine has shown similar effects in rat islets.

IL-1β not only induces NOS expression, but it also alters the expression of several other genes. Chen et al showed that IL-1β not only upregulated NOS, but it also upregulated the expression of certain chemokines such as monocyte

chemotractant protein 1 (20). This may play a role in insulitis in the recruitment of monocytes and other inflammatory cells. Another experiment on purified beta cells showed that IL-1β is able to upregulate superoxide dismutase, heme oxygenase and other free radical scavengers. This may be an attempt by the cell to protect it self from the nitric oxide induced by IL-1β (21). IL-1β was also able to downregulate PDX-1, GLUT 2 and PC2. These are all important proteins in the development and function of islets. IL-1β may alter the phenotype of islets making them less responsive to glucose.

Scarim et al tested the ability of rat insulinoma cells (RIN m5F) and rat islets to recover from different lengths of culture in the presence of IL-1β (19). This study shows that rat islets exposed to IL-1β for 24, 36 and 48 hours all have poor response to a glucose challenge. However, islets cultured for only 24 hours in IL-1β if washed immediately and incubated with a NOS inhibitor for 8 hours will regain normal insulin responses to a glucose challenge. Thirty-six and 48 hour cultures of rodent islets with IL-1β are not able to regain their normal function no matter how long they are incubated with NOS inhibitors. This article suggests that islet exposure to IL-1β for any longer than 36 hours will result in irreversible functional inhibition.

Inducible nitric oxide synthase plays an essential role in IL-1β induced damage to rodent islets. iNOS converts cellular L-arginine to nitric oxide, which targets mitochondrial respiration by inhibiting aconitase yielding lower ATP production (19). Nitric oxide can also react with oxygen free radical to generate peroxynitrite, which may be harmful to cells. In rat islets, IL-1β is adequate to induce NOS expression, in humans this may not be the case. Nitric oxide is an important molecule involved in neurotransmission, vasodilation (endothelial cells) and as stated previously it is secreted by macrophages because of its anti-microbial qualities. Nitric oxide in neural and endothelial cells is secreted by a constitutively expressed form of the enzyme. In macrophages and most mammalian cells, there exists an inducible form of nitric oxide synthase. Its expression is stimulated through cytokines and lipopolysaccharide signaling. Macrophages use nitric oxide as part of the body's innate immune system against fungal, bacterial, or other pathogens. Inducible nitric oxide synthase is a 37 kilobase gene consisting of 26 exons and 25

introns. The gene regulating elements contain binding sites for several transcription factors such as interferon response element-1 (IRE-1), NF-kB, Gas/STAT and several others.

## The effects of pro-inflammatory cytokines on human islets

Most of the early studies tested the effects of pro-inflammatory cytokines on rodent islets. Although important, it is hard to apply the results of experiments done on rodent tissue to human islets. In 1990, Rabinovitch and colleagues first tested the effects of IL-1β, IFNγ and TNFα on human islets in vitro (22). They found alone, that both IL-1β and TNFα were able to cause islet cell death. IFNγ had little effect on cell survival. However, all three cytokines together produced an increase in islet cell death that was more dramatic than any one cytokine alone. This "cocktail" drastically reduced the insulin content of cultured islets. Glucagon content of the islets was also decreased suggesting that cytokines were not beta cell specific. The author speculates that IFNγ may potentiate the effects of IL-1β and TNFα by increasing class two MHC expression and stimulating resident islet macrophages to produce more IL-1β which may amplify the effects. Later studies by Corbett et al tested whether or not iNOS and nitric oxide production mediated the islet cytotoxicity of cytokines (23). This study demonstrated that IL-1β alone at levels toxic to rodent islets was unable to stimulate nitric oxide production in human islets. IL-1β and IFNγ, when combined, were able to stimulate human islet nitric oxide production with TNFα potentiating the effect. More than one cytokine signal seems necessary for nitric oxide production in human islets. cGMP levels, an indicator of nitric oxide production, were also elevated in the exposed islets. Low concentrations of cytokines slightly stimulated insulin release. Yet, much higher concentrations of cytokines caused large nitric oxide levels and potent inhibition of islet function. $N^G$-monomethyl-L-arginine, or NMMA, a potent inhibitor of nitric oxide production by iNOS only partially prevents the functional effects of cytokines on human Islets. Other signaling and cellular mechanisms must still be occurring. Corbett suggests that nitric oxide does play a role in the inhibition, but other signaling such as that through JNK may be involved.

In rodent islets, IL-1β was able to cause DNA damage, and integrity damage. Delaney et al showed that the cytokine cocktail significantly increased the amount of DNA strand breakage six and nine days post culture in human islets (24). An increase in apoptotic cells was also noticed. The nitric oxide levels of exposed islets increased, but inhibition of iNOS did not decrease DNA strand breakage or the amount of apoptosis. This article provides evidence that the cytokines in question do induce DNA strand breakage. This effect occurs regardless of nitric oxide production. Alternative signaling such as that through ceramide, FasL, or caspases may be causing these effects.

Developing IDDM in human patients often causes disproportionately high proinsulin levels, which may serve as an early marker for the disease. Hostens et al monitored the proinsulin content and release of human islets exposed to IL-1β, IFNγ and TNFα (25). Culture together produced a decrease in cellular insulin content, while the total insulin in both the islet cells and culture media remained normal. More insulin must have been released into the media. The cytokines also caused an increase in media proinsulin levels while cellular levels were similar to those in controls. This effect was caused irrespective of nitric oxide formation. Proinsulin synthesis remained normal in cytokine induced islets, but conversion of proinsulin was markedly decreased. The authors showed a decrease in PC1 and PC2, the enzymes responsible for the conversion. It seems that the cytokines are able to decrease cellular insulin via two methods: by causing increased secretory activity resulting in high media insulin levels and by preventing the conversion of pro-insulin to insulin by altering PC1 and PC2. Cytokines may not alter cellular insulin by cytodestrucive methods. The drop may simply occur due to changes in the proinsulin converting mechanism. These changes are brought about by cytokine exposure. Ling et al also showed that IL-1β was able to reduce PC2 expression in purified rat islets (21).

Another study discovered that oxygen free radical production and aldehyde generation, not nitric oxide radicals, might be causing the cytotoxic effects of cytokines (26). The cytokine induced formation of oxygen free radicals such as

superoxide and the hydroxide radical increase lipid peroxidation resulting in the generation of toxic aldehydes. In this study, the cytokine cocktail significantly increased both nitric oxide levels and maliondialdehyde levels. Maliondialdehyde, or MDA, is the end product of oxygen free radical generated lipid peroxidation. Inhibition of nitric oxide production by NMMA did not prevent cytokine induced insulin and DNA reduction. Inhibition of free radical-lipid peroxidation by the lazaroid anti-oxidant drastically lessened the effects of cytokines on islet insulin and DNA content.

Karlsen et al were able to show that IFNγ and TNFα or a combination of all three cytokines in question were able to increase ICE mRNA expression in human islets (27). Interleukin 1 converting enzyme, is a crucial regulator of a pro-apoptotic cascade. Nitric oxide was not necessary for ICE expression, in fact it somewhat inhibited its expression. IFNγ seems required for ICE production. The author suggests that IFNγ mediates its effect through the transcription factor IRF-1 or Interleukin regulating factor one. This paper provides another signal cascade that may be involved in the cytokine signaling of IL-1β, IFNγ and TNFα.

## Species differences of rodent and human islets

Several studies tested the difference in susceptibility to islet injury between rodent and human pancreatic islets. Eizirik et al looked at possible difference between the effect on murine, rat and human islets by several islet cell stressors such as streptozotocin, alloxan, and nitric oxide generators (28). Both alloxan and streptozotocin caused inhibition of insulin release and apoptosis in islets obtained from mice or rats. These two islet toxins, however, had little effect on human islets. Sodium nitroprusside, a potent nitric oxide donor, was able to generate similar levels of nitric oxide in all three species of islets. Human islets were not affected, but both rat and mouse islets showed poor glucose stimulate insulin release and damage to cell integrity. The authors suggest that the one can not extrapolate and compare data obtained using rat islets directly back to human islets. Hadjivassilou performed a similar experiment in which both rat and human islets were exposed to several

chemical generators of free radicals and to the cytokine cocktail (29). Donors of the hydroxide radical and peroxynitrite produced little effect in both species. Nitric oxide donors did affect the rat islets alone. The three cytokine mixture induced nitric oxide production, DNA strand breakage and cell death in both species of islets.

**Role of macrophages in islet destruction**

Macrophages play a very important role in immunity. Macrophages are involved in antigen presentation as well as phagocytosis of antibody bound antigen. They also mediate cytotoxicity by synthesizing and secreting many molecules that may be detrimental to foreign pathogen or infected cells. Macrophages release cytokines (IL-1β), nitric oxide, proteolytic enzymes and various other factors (12). During infection, macrophages target to the site of inflammation where they are activated by interferon secreting Th cells.

Macrophages may also play an important role in the development of diabetes. Bach, in his review states that macrophages are the first mononuclear cell to enter islets during insulitis (3). Macrophages are also known to secrete large amounts on IL-1β when activated. Moreover, transfer of macrophages from diabetic NOD mice to pre-diabetic NOD mice can accelerate IDDM. Administering silica to kill macrophages prevents diabetes in NOD mice (30). Fehsel and colleagues suggest that inflammatory macrophages may be a source of nitric oxide during insulitis (31) resulting in islet cell inhibition and islet cell death. They showed that activated rat macrophages were able to induce DNA damage to rat islets. This damage was prevented by NMMA. Arnusch et al took this concept one step further (32) and tested whether TNFα and lipopolysaccharide (LPS), two factors which are known to activate macrophages, were able to induce resident islet macrophage production of IL-1β. They suggest that IL-1β in turn can cause beta cells to express iNOS, which mediates beta cell damage through nitric oxide. They showed that treatment of rat islet cells with TNFα and lipopolysaccharide (LPS) did result in a potent inhibition of islet function that was similar to the effects caused by the addition of exogenous IL-1β. IL-1β receptor agonist protein, IRAP-1, completely blocked the effects of TNFα

and LPS. Culture in conditions that destroy resident islet macrophages prevents the effect of TNFα and LPS on beta cell function. TNFα and LPS treatment was also shown to upregulate iNOS mRNA in rat beta cells. In a later publication, Arnusch et al suggested that human resident macrophages induced by TNFα, LPS and Interferon gamma can secrete IL-1 which mediates damage to human islets through iNOS expression (33). The authors describe an interesting explanation for the occurrence of IDDM. Infections may cause the infiltration of T cells and macrophages into the islet bodies. Stress and infection induce LPS secretion and TNFα and IFNγ release from Th cells. These factors may cause macrophages to activate and release beta cell toxins such as IL-1β and endogenously produced nitric oxide. The experiment carried out by the authors provides a clear role for resident macrophages in diabetes. A paper by Delaney and others examined whether exogenous nitric oxide produced by macrophages mediated beta cell DNA damage; or if endogenously produced nitric oxide in islets treated with IL-1β was sufficient to cause damage (34). They proved that both exogenous nitric oxide from a nitric oxide donor, and endogenous nitric oxide induced by IL-1β was able to nick beta cell DNA.

**Studies using isolated beta cells**

Islets consist of several cell types; endocrine cells, ductal cells, resident macrophages and other leukocytes. Previous cytokine studies used islets making it difficult to describe the effect of cytokines on beta cells. It is very likely that experiments involving islets have a complicated network of several cell types, like macrophages and beta cells that work together to cause the cytokine induced effects. Ling et al compared the effects of IL-1β on dissociated alpha, beta cells and islets (35). They noticed cultured islets exposed to IL-1β were reduced in size, had a loss of sphericity and a significant loss of both insulin and DNA content. In beta cells, however, cell morphology was similar to controls and DNA content was unaffected. Insulin content was decreased as well as insulin synthesis. A ratio of insulin recovery to DNA recovery was used to suggest that IL-1β caused beta cell degranulation. The authors also showed that the effects of IL-1β on individual beta cells were reversible with ample culture time. The effect on islets was not. IL-1β was cytodestructive to

islet cells, but merely cytostatic to beta cells. This is the first study showing a difference in islet and beta cell response to IL-1β. It also suggests more of a role for non-beta cells in cytokine controlled islet destruction. Further studies by Ling and others were able to show the changes IL-1β induced in the phenotype of rat beta cells (21). They noticed twenty-four hour culture of rat beta cells with IL-1β altered mRNA expression. Heme-oxygenase, mn superoxide dismutase, heat shock protein 70 and iNOS were all upregulated. PDX-1, GLUT 2, and PC2 were all downregulated. These genes are all important in controlling glucose stimulated insulin synthesis. Insulin synthesis was also decreased. Therefore, IL-1β changes beta cell phenotype making them less responsive to a glucose stimulated challenge. The beta cells were made more resistant to alloxan, streptozotocin, and various nitric oxide donors. Therefore, although the IL-1β made cells less responsive it did provide protective effects against beta cell toxins. This could be due to lower uptake of alloxan or streptozotocin due to lower GLUT 2 levels. In his review, Eizirik feels that the destruction of beta cell mass in diabetes involves a long drawn out battle between beta-cell destruction and beta cell repair with the former eventually winning the "war" (18). The authors note that this work should not be used to contradict work done on islet cells (21). In islets, other cells may help cause a larger accumulation of nitric oxide causing necrosis. Some other research suggests that the upregulation of mn superoxide dismutase, heme-oxygenase 1 and HSP-70 may be a protective response to the IL-1β evoked nitric oxide production (36). Hoorens et al compared the effect of IL-1β in concert with IFNγ on both rat and human beta cells (37). IL-1β, in this study failed to become cytotoxic after 9 days culture, whereas, IL-1β and IFNγ induced apoptosis in both rat and human beta cells. Necrosis was only noticed in rodent islets. iNOS inhibitors such as L-methylarginine prevented rodent beta cell necrosis but had no effect on apoptosis. Necrosis seems to be nitric oxide dependent while apoptosis seems to be independent of its production. They also cultured rat beta cells in increasing densities and found that IL-1β was now able to induce nitric-oxide dependent necrosis. They hypothesized that the failure of IL-1β to cause cytotoxic effects in beta cells was due to the increased distance between cells. This

makes it difficult to generate high enough nitric oxide concentrations around beta cells to cause damage.

### Therapeutic intervention for type 1 diabetes

Since it appears pro-inflammatory cytokines are able to mediate damage to human islets and they may be involved in the pathogenesis of IDDM, attempts must be made at preventing these cytokine induced effects. One group discovered that human islets transfected with high levels of interleukin-1 receptor agonist protein, IRAP-1, are able to prevent the cytostatic effects, nitric oxide production and caspase-3 activity caused by IL-1β (38). The Rabinovitch lab transfected human islets with BCL-2, an anti-apoptotic protein (39). This transfection prevented cytokine induced inhibition of islets and partially prevented islet DNA damage and lipid peroxidation. Another group showed that over-expression of BCL-2 in a rat insulinoma cell line produced similar effects (40). The transfected cell line had a reduction in both cytokine induced apoptosis and necrosis.

Corbett et al suggest that iNOS inhibitors may provide islet protection since they will drastically reduce nitric oxide mediated damage (41). NOS inhibitors like $N^G$-monomethyl-L-arginine (NMMA), and N-ω-nitro-L-arginine methyl ester (NAME) are not the best choice because they also prevent cNOS activity, which is crucial in maintaining proper blood pressure and vasodilation as well as for neurotransmission(18). Corbett suggests that aminoguanidine could be used since it targets iNOS with 40 times greater affinity then cNOS. Other groups have used IGF-1 and 2 with modest results (42,43). It seems premature to attempt to prevent the effects of cytokines before they are fully understood. Increased attention should be placed on deciphering the signaling involved in cytokine induced islet damage. This will lead to a better understanding of where intervention can take place.

## Islet transplantation- a real alternative for treating type 1 diabetes

The most likely solution to diabetes related complications is the transplantation of insulin producing tissue into inflicted patients. This physiological approach of restoring the insulin producing tissue in diabetics may allow for precise glycemic control preventing IDDM related incidences. Initially, whole pancreas transplants were performed (44). Since the first whole pancreas transplant, significant improvement has been made in this area and pancreas organ transplantation is now considered an efficacious treatment of diabetes. There are however several drawbacks to this therapy. First, whole pancreatic transplants involve very invasive surgery, which may lead to complications causing a difficult recovery and even the risk of mortality. Moreover, pancreas transplant requires life long immunosuppresion to prevent graft rejection and the potential recurrence of an autoimmune response towards the donor pancreatic beta cells. Another treatment strategy is required.

Early in this century, it became known that it was actually beta cells from the islets of Langerhans that were involved in regulating glucose metabolism through the production and synthesis of insulin. This lead to the idea of transplanting islet cells into diabetic patients rather than the intact pancreas. Islet transplantation was thought to be a more attractive solution for treatment of diabetes than whole pancreas transplant for several reasons. Islet transplantation can be achieved using much less invasive surgery. Isolated islets can also be cryopreserved and stored for long periods. Islet cells can be manipulated in vitro, prior to transplant, allowing us to possibly reduce their immunogenicity. Different culture conditions, gene therapy, removal of donor class two MHC bearing cells, and immuno-isolation devices have all been applied to islet transplantation in an attempt to prevent the need for immunosuppressive drug regimes. Furthermore, islet cells can also be manipulated in vitro to improve graft function prior to transplant.

The first successful attempt at experimental islet transplantation was reported by Ballinger and Lacy (44). They transplanted isogeneic rat islets into the peritoneal

cavity and thigh muscle of chemically induced diabetic rats. As islet transplantation progressed in small animal models, it was also attempted in larger animal models. The success that was achieved in rodents was not reproducible in larger animals. It took several decades of research to achieve consistent success in clinical islet transplantation. One reason for this was that early isolation procedures for rodent islets were ineffective for the dense, fibrous pancreas found in larger species. It became clear that better isolation strategies were required. More efficient means of digesting large mammalian pancreas were subsequently developed when collagenase, the enzyme used to digest and isolate islet cells, was introduced directly into the pancreatic duct by injection or continuos perfusion. These procedures allowed for the more effective delivery of the enzyme to the acinar tissue resulting in greater separation of islets from surrounding exocrine tissue. Unfortunately, the lack of consistent and effective lots of enzymes used in the isolation of canine and human islets prevented the recovery of large numbers of viable islets, thus making it hard for the field of islet transplantation to progress. The development of purified Liberase enzyme blends by the Boehringer-Manheim Corporation eliminated lot to lot variability and improved islet isolations in larger animals. However, even when isolation procedures were perfected and were able to provide high quality and quantity of islets, islet transplantation was unable to achieve consistent success. In fact, in the 305 total islet transplants performed world wide between 1974 and 1996 only 33 recipients remained insulin independent for longer than 1 week, and only 25 patients were insulin independent after one year post transplant. It was not until 2000, when the “Edmonton Protocol” developed at the University of Alberta was able to show, using a gluco-corticoid free immunosuppressive drug regimen, that islet transplantation can allow for the consistent restoration of euglycemia in an insulin independent manner. This study has shown that islet transplantation is a viable option to pancreatic organ transplantation. It should be noted, like whole pancreas transplantation, current islet transplantation also requires chronic immunosuppression. However, because we have the ability to manipulate islets in vitro prior to transplant there is the possibility of removing islet immunogenecity such that immunosuppression is no longer required.

A major drawback of islet transplantation is the shortage of insulin producing tissue. If this therapy is to ever be used as an alternative to insulin then adequate human islet tissue must be procured. However, this seems very unlikely. In the United States, there are 30,000 new cases of IDDM every year, yet only 3000 cadaver pancreases are obtained annually (45). According to Health Canada there are also approximately 6000 new cases of type 1 diabetes annually in Canada. In order to overcome this supply problem, insulin producing tissue from a number of abundant and accessible sources are being considered for clinical transplantation. Researchers have looked into porcine (46), fish brockman bodies (47)and bovine (48) islets as well as trying to genetically alter insulin producing cell lines. Porcine islets probably are the best-suited alternative source of islet tissue (49). Pigs breed quickly and give birth to large litters. Porcine insulin is also structurally similar to that of man, in fact porcine insulin has been used as a source for insulin therapy in humans for many years now (50).

Early work with porcine islet cells was performed using adult pigs. Factors such as age, breed and quality of organs adversely affect the final yield, and once isolated adult porcine islets are fragile and difficult to maintain in culture (46). Further research was able to show that collagenase digested fetal porcine pancreas produced islet like cell clusters which were able to cure diabetes in nude mice (51). However, the fetal islet-like clusters showed a poor responsiveness to a glucose challenge. Other groups have looked into using neonatal pigs as a tissue source for islet therapy. Islets isolated from neonatal pigs have tremendous growth potential, contain numerous well-granulated insulin and glucagon positive cells and exhibit the metabolic capacity to restore euglycemia in nude mice (52). Unlike porcine fetal islet like cell clusters, the neonatal porcine islet cells showed a clear response when presented with a glucose challenge. Neonatal tissue seems to be the best avenue available for a potential xenograft treatment for insulin dependent diabetes mellitus.

This leads to the second main complication in using islet therapy as a potential cure for diabetes. Islets given to inflicted individuals with type 1 diabetes are faced with a potent immune assault. Diabetic patients have lost their beta cells mass by an autoimmune rejection. Even though this attack may have occurred many years

previous it is still very likely that this autoimmune response can be activated against transplanted beta cells (45). There is evidence suggesting that autoimmune rejection may attack allogeneic tissue as well as xenografted beta cells. A second type of response, allograft rejection, usually occurs due to major (MHC) and minor (minor histocompatibility antigens) differences between cell surface markers/antigens of donor and recipient tissue. This rejection is usually mediated by T cells and requires two signals to occur (50):

1. TCR interaction with its specific graft antigen
2. Co-stimulation provided by APC and T cell co-receptors

These two signals can be achieved via two main pathways as described by Rayat (50); the direct pathway, in which host T cells receive co-stimulation from donor APC's or through an indirect pathway in which host T cells receive co-stimulation from host APC's that have processed graft tissue antigens. Allorejection occurs because of the presence of both pathways. In contrast, xenograft rejection seems to occur predominantly via the indirect pathway. Studies by Gill et al (53) clearly support this statement. A study by Simeonovic et al (54) also suggests that allograft and xenograft rejection occur through different means. This study showed that allograft rejection was characterized with a rise in CD8 and CD4 positive T cells. This rejection was also closely associated with a Th1 response of high levels of pro-inflammatory cytokines such as IL-2, IFN gamma and IL-3. On the other hand, xenograft rejection shows a clear dependence on CD4 positive T cells and eosinophils. Large rises in Th2 cytokines such as IL-4 and IL-5 were noticed in xenorejection. The literature clearly shows that allogeneic and xenogeneic graft rejection occur via different mechanisms, therefore, different anti-rejection therapies must be developed for each if we are to improve the success of islet transplantation.

The indirect T cell mediated xenograft rejection is just one of several immunological barriers presented by discordant tissue. Perhaps, of even more importance for xenotransplantation, is the occurrence of humoral-mediated graft rejection. In this process, naturally occurring xenoreactive antibodies to cell surface antigens on transplanted tissue are believed to evoke a complement cascade capable of destroying the tissue (55). In whole organs, this pathway for rejection is able to

destroy the endothelial cells lining the graft resulting in extensive hemorrhaging and thrombosis of graft tissue. This type of response occurs minutes to hours post operation and is unable to be controlled by immunosuppressive drugs (55). There was some thought that since islet cells are not primarily vascularized grafts they would not be susceptible to complement mediated hyperacute rejection in the same manner as vascularized organ transplants. However, research by Gray et al has shown that xenogeneic islets transplanted into cynomolgus monkeys undergo extensive destruction within six hours of transplant (56). This rejection has histological features typical of hyperacute rejection. This research suggests that hyperacute rejection may occur in islet grafts. In porcine tissue, the cell surface antigen that the human preformed antibodies are reacting to is a carbohydrate referred to as Gal-alpha- (1,3)- Gal –beta (1,4) GlcNac, or in short the alpha-Gal epitope (57,58). Humans lack the enzyme –alpha galactosyltransferase- that is able to glycosylate porcine tissue with this sugar moiety. Therefore, bacterial infections presenting pseudo alpha Gal epitope have allowed for the presence of naturally occurring antibodies against the porcine Gal-alpha- (1,3)- Gal –beta (1,4) GlcNac (59). The epitope is present on all porcine tissue. Rayat et al showed that this alpha Gal epitope is also present on neonatal insulin and glucagon positive cells (60). This work was able to show that neonatal porcine islet cell aggregates, both Gal positive and Gal negative cells, were susceptible to human antibody complement mediated lysis in vitro (60). This study shows, that like solid porcine organs, porcine neonatal islet cells may also be susceptible to complement mediated cell lysis. Before this can be confirmed, in vivo studies must be performed. Moreover, this study indicates, since Gal negative cells were also lysed, that Gal expression may not be critical for cell lysis. Several attempts have been made at developing tissue lacking its expression. However, a lack of porcine embryonic stem cells has made this very difficult (55). Perhaps, another approach is to develop a porcine line expressing human complement regulatory sub units on their endothelial surface (49,50,55). This would allow transplanted pig tissue to down regulate and prevent complement activation thus possibly circumventing complement mediated cell lysis.

The last main concern with the use of porcine tissue as a source of alternative islet cells deals with the transfer of retrovirus and other infections from donor to human recipients. Pigs contain retroviral sequences that are stably incorporated within the genomes of porcine cells. There is concern that in porcine xenografts there may be cross species transmission of this porcine endogenous retrovirus, PERV, to human recipients. There are several studies that have shown the porcine xenografts may transmit PERV to Scid mouse recipients (61,62). However, there are also several studies reviewing the transmission of PERV from porcine xenograft transplants in human recipients. These studies were unable to detect markers for PERV infection in any of the human recipients (63-65). There is still debate as to whether transmission of porcine endogenous retrovirus to human recipients will be a consequence of porcine xenograft transplantation.

Transplantation of discordant xenogeneic tissue, the most attractive alternative source of islet cells, was compared to an onion by David G. White (66). He states that "once you peel away one layer of immunological problems, another set lie beneath." As stated earlier, previous research by Rayat (60) indicated that the first layer of immune problems with porcine tissue transplantation may be complement mediated cell lysis via xenoreactive antibodies. Research from our lab attempted to use immunoisolation devices, alginate microcapsules specifically, to prevent complement mediated cell lysis of neonatal porcine islet cells in vitro (67). Neonatal porcine islet cells, NPIC, were encapsulated using the alginate. Non-encapsulated islets, and encapsulated islets were cultured with fresh human serum and insulin, DNA, and glucose stimulation studies were performed. Significant insulin and DNA losses in non-encapsulated islets were observed, while encapsulated islets had levels similar to controls(60). The non-encapsulated islets also had a very poor glucose stimulation index after culture in the fresh serum(60). In contrast, encapsulated islets again showed glucose stimulation similar to that of controls. Rayat et al outline a role for encapsulated NPIC in the treatment of diabetes as these capsules may be able to prevent complement mediated cell lysis in vivo.

The next layer of our "onion" involves both CD4+ and CD8+ T cells. Research by Murray et al in conjunction with our lab indicates that human peripheral blood leukocytes cultured with NPIC can proliferate and respond (68). However, this proliferation is mounted primarily by CD4+ T cells recognizing the class two swine leukocyte antigen (SLA). This research suggests that human CD8+ T cells, that are able to lyse neonatal spleen and endothelial cells, may not be able to proliferate and respond to NPIC. Treatment of NPIC with cytokine supernatant does increase the density of SLA class one expression but islet cell lysis is not improved. Addition of lectin-PHA does increase NPIC death when cultured with primed CD8+ cells. It seems that human CD8+ PBL's may not be able to respond to NPIC due to their failure to form tight conjugates with the islets. This is due to the lack of adhesion molecules on NPIC. Human natural killer cells also fail to lyse NPIC. This research reveals that xenogeneic islet grafts may be able to circumvent CTL-mediated lysis. CD4+ T cells do respond strongly to NPIC. These cells may be able to damage xenogeneic grafts via cytokine production or recruitment of other immune cells etc.

## Cytokines-the next barrier to porcine islet transplantation

The literature shows that inflammatory cytokines may be involved in the pathogenesis of diabetes. IL-1β, IFNγ and TNFα are able to inhibit function, induce DNA damage and cause apoptosis/necrosis in both rat and human islet cell preps in vitro. Since porcine islets seem to be the most likely tissue source for islet transplantation, it is crucial to determine the effects of human pro-inflammatory cytokines on NPIC. If xeno-islet transplantation is to work, we need to be able to prevent any damage that may be caused by these potent signaling molecules. Encapsulation of porcine neonatal islet cells may allow us to prevent complement mediated cell lysis. Unfortunately, encapsulation excludes most molecules over 150 kilodaltons in size. Therefore, it is likely cytokines will still be able to access encapsulated porcine islets since they are smaller than the molecular weight cut off of alginate capsules. An article by Piro et al evaluated the effects of human cytokines on both bovine and human islets (69). This study showed that in contrast to human islets, bovine islet insulin release was not affected after 24 hour culture with human

IL-1β, IFNγ and TNFα. Human islets exposed to cytokines showed an increase in both early and late apoptosis where as bovine islets had levels of apoptosis similar to controls. Reverse Transcription- PCR showed elevated iNOS and BAX/BCL-2 ratios in exposed human islets indicating a pro-apoptotic morphology (70). Bovine islets had similar iNOS levels both in culture with and without cytokines. The ratio of BAX to BCL-2 also remained constant in both treated and non-treated bovine islets. Human cytokines do not seem able to affect bovine islets. We must remember that there does seem to be a difference between species susceptibility to human cytokines.

Our next step in xenotransplantation using neo-natal porcine islet cells must be an examination of the effects of human Th1 cytokines on these very cells. We may be able to prevent humoral-mediated xenograft rejection using immunoisolation. NPIC may resist cell lysis mediated by human CD8+ T cells since human CD8+ T cells may not respond to NPIC. The next step for porcine islet transplantation must be first to determine the effects of cytokines on NPIC, and then to provide methods to circumvent any harmful or detrimental effects. I hypothesize that exposure of neonatal porcine islet cells to the human pro-inflammatory cytokines, IL-1β, IFNγ and TNFα, will likely result in cell death and reduced glucose stimulated insulin secretion.

## References

1. Eisenbarth GS. - Type I diabetes mellitus. A chronic autoimmune disease. - N Engl J Med 1986 May 22;314(21):1360-8. 1360.
2. Noorchashm H, Kwok W, Rabinovitch A, Harrison LC. - Immunology of IDDM. - Diabetologia 1997 Oct;40 Suppl 3(8):B50-7. B50.
3. Bach JF. - Insulin-dependent diabetes mellitus as an autoimmune disease. - Endocr Rev 1994 Aug;15(4):516-42. 516.
4. Rabinovitch A, Suarez-Pinzon WL. - Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. - Biochem Pharmacol 1998 Apr 15;55(8):1139-49. 1139.
5. Anonymous- Implications of the Diabetes Control and Complications Trial. American Diabetes Association. - Diabetes 1993 Nov;42(11):1555-8. 1555.
6. Nathan DM. - Long-term complications of diabetes mellitus. - N Engl J Med 1993 Jun 10;328(23):1676-85. 1676.
7. Tisch R, McDevitt H. - Insulin-dependent diabetes mellitus. - Cell 1996 May 3;85(3):291-7. 291.
8. Kuby J. Various Chapters. In: Immunology, Allen D., ed. New York, W.H. Freeman and Company, 1998;
9. Baekkeskov S, Aanstoot HJ, Christgau S, et al. - Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. - Nature 1990 Sep 13;347(6289):151-6. 151.
10. Kaufman DL, Clare-Salzler M, Tian J, et al. - Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. - Nature 1993 Nov 4;366(6450):69-72. 69.
11. Daniel D, Gill RG, Schloot N, Wegmann D. - Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. - Eur J Immunol 1995 Apr;25(4):1056-62. 1056.
12. Mandrup-Poulsen T. - The role of interleukin-1 in the pathogenesis of IDDM. - Diabetologia 1996 Sep;39(9):1005-29. 1005.

13. Rabinovitch A. - Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? - Diabetes 1994 May;43(5):613-21. 613.

14. Mandrup-Poulsen T, Bendtzen K, Nerup J, Egeberg J, Nielsen JH. - Mechanisms of pancreatic islet cell destruction. Dose-dependent cytotoxic effect of soluble blood mononuclear cell mediators on isolated islets of Langerhans. - Allergy 1986 May;41(4):250-9. 250.

15. Mandrup-Poulsen T, Bendtzen K, Nerup J, Dinarello CA, Svenson M, Nielsen JH. - Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. - Diabetologia 1986 Jan;29(1):63-7. 63.

16. Sandler S, Andersson A, Hellerstrom C. - Inhibitory effects of interleukin 1 on insulin secretion, insulin biosynthesis, and oxidative metabolism of isolated rat pancreatic islets. - Endocrinology 1987 Oct;121(4):1424-31. 1424.

17. Southern C, Schulster D, Green IC. - Inhibition of insulin secretion by interleukin-1 beta and tumour necrosis factor-alpha via an L-arginine-dependent nitric oxide generating mechanism. - FEBS Lett 1990 Dec 10;276(1-2):42-4. 42.

18. Eizirik DL, Flodstrom M, Karlsen AE, Welsh N. - The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. - Diabetologia 1996 Aug;39(8):875-90. 875.

19. Scarim AL, Heitmeier MR, Corbett JA. - Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1beta. - Endocrinology 1997 Dec;138(12):5301-7. 5301.

20. Chen MC, Schuit F, Eizirik DL. - Identification of IL-1beta-induced messenger RNAs in rat pancreatic beta cells by differential display of messenger RNA. - Diabetologia 1999 Oct;42(10):1199-203. 1199.

21. Ling Z, Van De Casteele M, Eizirik DL, Pipeleers DG. Interleukin-1beta-induced alteration in a beta-cell phenotype can reduce cellular sensitivity to conditions that cause necrosis but not to cytokine-induced apoptosis. Diabetes 2000; 49: 340.

22. Rabinovitch A, Sumoski W, Rajotte RV, Warnock GL. - Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. - J Clin Endocrinol Metab 1990 Jul;71(1):152-6. 152.

23. Corbett JA, Sweetland MA, Wang JL, Lancaster JR, McDaniel ML. - Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. - Proc Natl Acad Sci U S A 1993 Mar 1;90(5):1731-5. 1731.

24. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL. - Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. - Endocrinology 1997 Jun;138(6):2610-4. 2610.

25. Hostens K, Pavlovic D, Zambre Y, et al. - Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release. - J Clin Invest 1999 Jul;104(1):67-72. 67.

26. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, et al. - Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production. - J Clin Endocrinol Metab 1994 Oct;79(4):1058-62. 1058.

27. Karlsen AE, Pavlovic D, Nielsen K, et al. - Interferon-gamma induces interleukin-1 converting enzyme expression in pancreatic islets by an interferon regulatory factor-1-dependent mechanism. - J Clin Endocrinol Metab 2000 Feb;85(2):830-6. 830.

28. Eizirik DL, Pipeleers DG, Ling Z, Welsh N, Hellerstrom C, Andersson A. - Major species differences between humans and rodents in the susceptibility to pancreatic beta-cell injury. - Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9253-6. 9253.

29. Hadjivassiliou V, Green MH, James RF, Swift SM, Clayton HA, Green IC. - Insulin secretion, DNA damage, and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite, and cytokines. - Nitric Oxide 1998;2(6):429-41. 429.

30. Shimada A, Takei I, Maruyama T, et al. - Acceleration of diabetes in young NOD mice with peritoneal macrophages. - Diabetes Res Clin Pract 1994 Jun;24(2):69-76. 69.

31. Fehsel K, Jalowy A, Qi S, Burkart V, Hartmann B, Kolb H. - Islet cell DNA is a target of inflammatory attack by nitric oxide. - Diabetes 1993 Mar;42(3):496-500. 496.

32. Arnush M, Scarim AL, Heitmeier MR, Kelly CB, Corbett JA. - Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. - J Immunol 1998 Mar 15;160(6):2684-91. 2684.

33. Arnush M, Heitmeier MR, Scarim AL, Marino MH, Manning PT, Corbett JA. - IL-1 produced and released endogenously within human islets inhibits beta cell function. - J Clin Invest 1998 Aug 1;102(3):516-26. 516.

34. Delaney CA, Green MH, Lowe JE, Green IC. - Endogenous nitric oxide induced by interleukin-1 beta in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. - FEBS Lett 1993 Nov 1;333(3):291-5. 291.

35. Ling Z, In't VP, Pipeleers DG. - Interaction of interleukin-1 with islet beta-cells. Distinction between indirect, aspecific cytotoxicity and direct, specific functional suppression. - Diabetes 1993 Jan;42(1):56-65. 56.

36. Ling Z, Chen MC, Smismans A, et al. - Intercellular differences in interleukin 1beta-induced suppression of insulin synthesis and stimulation of noninsulin protein synthesis by rat pancreatic beta-cells. - Endocrinology 1998 Apr;139(4):1540-5. 1540.

37. Hoorens A, Stange G, Pavlovic D, Pipeleers D. - Distinction between interleukin-1-induced necrosis and apoptosis of islet cells. - Diabetes 2001 Mar;50(3):551-7. 551.

38. Giannoukakis N, Rudert WA, Ghivizzani SC, et al. - Adenoviral gene transfer of the interleukin-1 receptor antagonist protein to human islets prevents IL-1beta-induced beta-cell impairment and activation of islet cell apoptosis in vitro. - Diabetes 1999 Sep;48(9):1730-6. 1730.

39. Rabinovitch A, Suarez-Pinzon W, Strynadka K, et al. Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. Diabetes 1999; 48: 1223.

40. Saldeen J. Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. Endocrinology 2000; 141: 2003.

41. Corbett JA, McDaniel ML. - Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. - Diabetes 1992 Aug;41(8):897-903. 897.

42. Harrison M, Dunger AM, Berg S, et al. Growth factor protection against cytokine-induced apoptosis in neonatal rat islets of Langerhans: role of Fas. FEBS Lett 1998; 435: 207.

43. Hill DJ, Petrik J, Arany E, McDonald TJ, Delovitch TL. - Insulin-like growth factors prevent cytokine-mediated cell death in isolated islets of Langerhans from pre-diabetic non-obese diabetic mice. - J Endocrinol 1999 Apr;161(1):153-65. 153.

44. Nason RW, Rajotte RV, Warnock GL. - Pancreatic islet cell transplantation: past, present and future. - Diabetes Res 1988 Jan;7(1):1-11. 1.

45. Weir GC, Bonner-Weir S. Islet transplantation as a treatment for diabetes. J Am Optom Assoc 1998; 69: 727.

46. Ricordi C, Socci C, Davalli AM, et al. - Isolation of the elusive pig islet. - Surgery 1990 Jun;107(6):688-94. 688.

47. Wright JR, Polvi S, MacLean H. - Experimental transplantation with principal islets of teleost fish (Brockmann bodies). Long-term function of tilapia islet tissue in diabetic nude mice. - Diabetes 1992 Dec;41(12):1528-32. 1528.

48. Marchetti P, Giannarelli R, Cosimi S, et al. - Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. - Diabetes 1995 Apr;44(4):375-81. 375.

49. White DJ. - Transplantation of organs between species. - Int Arch Allergy Immunol 1992;98(1):1-5. 1.

50. Rayat GR, Rajotte RV, Korbutt GS. Potential application of neonatal porcine islets as treatment for type 1 diabetes: a review. Ann N Y Acad Sci 1999; 875: 175.

51. Korsgren O, Sandler S, Landstrom AS, Jansson L, Andersson A. - Large-scale production of fetal porcine pancreatic isletlike cell clusters. An experimental tool for studies of islet cell differentiation and xenotransplantation. - Transplantation 1988 Mar;45(3):509-14. 509.

52. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119.

53. Gill RG, Wolf L, Daniel D, Coulombe M. - CD4+ T cells are both necessary and sufficient for islet xenograft rejection. - Transplant Proc 1994 Jun;26(3):1203. 1203.

54. Simeonovic CJ, Townsend MJ, Morris CF, et al. - Immune mechanisms associated with the rejection of fetal murine proislet allografts and pig proislet xenografts: comparison of intragraft cytokine mRNA profiles. - Transplantation 1999 Apr 15;67(7):963-71. 963.

55. Bradley JA. - Overcoming the immunological barriers to xenotransplantation. - Transplantation 1999 Jul 15;68(1):9-11. 9.

56. Hamelmann W, Gray DW, Cairns TD, et al. - Immediate destruction of xenogeneic islets in a primate model. - Transplantation 1994 Nov 27;58(10):1109-14. 1109.

57. Jurd KM, Lee J, Cairns T, Hunt BJ. - Effect of Gal alpha 1,3 Gal-beta 1,4 GlcNAc and complement depletion on haemostatic activation in an in vitro model of the pig-to-human xenograft reaction. - Transplant Proc 1996 Apr;28(2):637-8. 637.

58. Galili U, Rachmilewitz EA, Peleg A, Flechner I. - A unique natural human IgG antibody with anti-alpha-galactosyl specificity. - J Exp Med 1984 Nov 1;160(5):1519-31. 1519.

59. Galili U. - Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans. - Immunol Today 1993 Oct;14(10):480-2. 480.

60. Rayat GR, Rajotte RV, Elliott JF, Korbutt GS. - Expression of Gal alpha(1,3)gal on neonatal porcine islet beta-cells and susceptibility to human antibody/complement lysis. - Diabetes 1998 Sep;47(9):1406-11. 1406.

61. Deng YM, Tuch BE, Rawlinson WD. - Transmission of porcine endogenous retroviruses in severe combined immunodeficient mice xenotransplanted with fetal porcine pancreatic cells. - Transplantation 2000 Oct 15;70(7):1010-6. 1010.

62. van der Laan LJ, Lockey C, Griffeth BC, et al. - Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice. - Nature 2000 Sep 7;407(6800):90-4. 90.

63. Dinsmore JH, Manhart C, Raineri R, Jacoby DB, Moore A. - No evidence for infection of human cells with porcine endogenous retrovirus (PERV) after exposure to porcine fetal neuronal cells. - Transplantation 2000 Nov 15;70(9):1382-9. 1382.

64. Heneine W, Tibell A, Switzer WM, et al. - No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. - Lancet 1998 Aug 29;352(9129):695-9. 695.

65. Paradis K, Langford G, Long Z, et al. - Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. - Science 1999 Aug 20;285(5431):1236-41. 1236.

66. White DJ. hDAF transgenic pig organs: are they concordant for human transplantation? XENO 1996; 4: 50.

67. Rayat GR, Rajotte RV, Ao Z, Korbutt GS. Microencapsulation of neonatal porcine islets: protection from human antibody/complement-mediated cytolysis in vitro and long-term reversal of diabetes in nude mice. Transplantation 2000; 69: 1084.

68. Murray AG, Nelson RC, Rayat GR, Elliott JF, Korbutt GS. - Neonatal porcine islet cells induce human CD4+, but not CD8+, lymphocyte proliferation and resist cell-mediated cytolytic injury in vitro. - Diabetes 1999 Sep;48(9):1713-9. 1713.

69. Piro S, Lupi R, Dotta F, et al. - Bovine islets are less susceptible than human islets to damage by human cytokines. - Transplantation 2001 Jan 15;71(1):21-6. 21.

70. Alberts B., Bray D., Lewis J., Raff M., Roberts K., Watson J.D. Molecular Biology of THE CELL. London: Garland Publishing Inc., 1994.

# Exposure of Neonatal Porcine Islets to Human Cytokines Reduces Cellular Insulin Content but not Functional Viability

## Introduction

A significant focus of diabetes research has been to develop better methods of treatment, which can prevent long-term complications of diabetes. The replacement of endocrine tissue with islet transplantation is an attractive approach since it offers a physiological means for precise restoration of euglycemia in patients with insulin dependent diabetes mellitus. The "Edmonton Protocol", using a gluco-corticoid free immunosuppressive drug regimen, has shown that islet transplantation can allow for the tight restoration of euglycemia in an insulin independent manner (1). Unfortunately, this protocol requires two to four donor pancreases for one transplant to achieve a critical islet mass to allow for insulin independence. For islet transplantation to become a commonly used practice in the treatment of type 1 diabetes, the problem of a limited supply of donor organs must be solved. A shortage in human donor organs has led to the research of other potential sources of islet tissue. Porcine islet tissue (2,3), bovine islet tissue (4), fish brockman bodies (5), genetically engineered insulin secreting cell lines (6,7) and in vitro expansion of human fetal (8) or adult islet cells (9) have all been examined. Porcine islets represent a suitable alternative source of insulin producing tissue. Pigs are inexpensive, they give birth to large litters and they exhibit physiological and morphological characteristics similar to humans. We have developed a procedure that allows the use of neonatal porcine pancreas as a source of large numbers of viable islet cells with reproducible and defined cellular composition (2). Neonatal porcine islets present an attractive source of insulin producing tissue for clinical transplantation due to their unlimited supply and inherent ability to proliferate and differentiate.

An obstacle with discordant porcine xenotransplantation is the potent immune response it may evoke from human recipients. Antibody-complement mediated cell lysis may occur after transplantation causing rapid destruction of porcine islets (10,11). Research from our lab has shown that encapsulating neonatal porcine islet

tissue in alginate microcapsules may allow them to circumvent complement mediated rejection in vivo (12). T cell mediated immune responses also represent a barrier to successful xenotransplantation. Murray et al showed that both human CD8+ and NK cells were not able to respond to porcine islet tissue. Therefore, porcine islets may be able to resist human cell mediated lysis (13).

Another immunological barrier to successful porcine xenotransplantation may be presented by recipient pro-inflammatory cytokines such as IL-1β, IFNγ, and TNFα. The autoimmune response leading to type 1 diabetes involves the infiltration of T-cells, macrophages and other lymphoid cells into the islet cells of the patient (14). It has been suggested that cytokines may be delivered in a site specific manner, by these infiltrating cells, to beta cells resulting in poor function and cytotoxic effects (15). Moreover, the combination of recombinant human IL-1β, IFNγ, TNFα have been shown to cause both functional and structural effects in rodent (16-19) and human (20,21) islet cell cultures in vitro.

In my study I tested the effects of the human pro-inflammatory cytokines IL-1β, TNFα and IFNγ on neonatal porcine islet cell preparations and human islet cell cultures. The aims of this study were to evaluate any functional effects of human cytokines on neonatal porcine islet cells. I tested the effects of cytokine exposure on islet viability, as well as for signs of cell death. Exposure of neonatal porcine islet cells to the pro-inflammatory human cytokines, IL-1β, TNFα and IFNγ, will most likely result in cell death and reduced glucose stimulated insulin secretion.

## Materials and Methods

### Preparation of neonatal porcine islets

The method used to prepare porcine neonatal islet cell (NIC) aggregates has been previously described (2). Landrace-Yorkshire neonatal pigs ages 1-3 days of either sex were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreases were removed, cut into small pieces, and digested with 2.5mg/mL collagenase (Sigma type V, St. Louis, MO). After filtration through a 500μm screen, the tissue was cultured for 5-7 days in HAM's F-10 medium (Gibco, Burlington Canada) containing 10mmol/l glucose, 50μmol/l isobutylmethylxanthine

(ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10mmol/l nicotinamide (BDH Biochemical, Poole, U.K.), 100 U/ml penicillin, and 100 μg/ml streptomycin.

### Isolation of human islets

Adult human pancreases were obtained, with informed consent of relatives, from six brain dead heart-beating kidney donor subjects. Tissue procurement and experimental protocols were approved by the human ethics committee of University of Alberta Hospitals. Islets were isolated from the harvested pancreas by intraductal collagenase distention and digestion then purified on ficoll gradients, as previously described by Warnock et al (22). Islets were cryopreserved in a manner previously described (23) using DMSO as the cryoprotectant. Briefly islets were suspended in medium 199 (Gibco, Grand Island, NY) supplemented with 10%(v/v) fetal bovine serum, 25 mmol/l HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and the concentration of DMSO was added in a stepwise manner (0.67, 1.0, and 2.0 mol/l) over a period of thirty minutes. All samples were then supercooled to –7.4 °C, nucleated, and, after release of the latent heat of fusion, slowly cooled at 0.25 °C/min to -40 °C and then plunged into liquid nitrogen for low temperature storage. After storage, human islets were thawed rapidly as described earlier (23). Islets were brought quickly to 0 °C in a 37 °C water bath at a rate of 200 °C/min. After centrifugation and removal of the supernatant, the intracellular DMSO was removed by the addition of 0.75 mol/l sucrose for 30min. (0 °C), followed by stepwise addition of supplemented M199 solution (5 min. intervals over a 30 min. period). Following thaw, islets were cultured in HAM's F-10 medium at 37°C under 95% air/ 5% $CO_2$ for 48 hours.

### Islet culture and cytokine exposure

Cultured porcine NIC aggregates and human islets were washed twice with HAM's F-10 medium. Representative aliquots were taken to assess insulin and DNA contents. Porcine NIC preparations were then aliquoted evenly into four 100mm x 15mm non-treated tissue culture plates (Fischer Scientific). Two plates served as

experimental groups and were exposed to cytokines whereas two plates served as controls and received no treatment. Human islets were also aliquoted evenly into two 100mm x 15mm non-treated tissue culture plates (Fischer Scientific) due to less tissue volume. Again, one plate served as a control and one plate served as an experimental group. The experimental groups were exposed to recombinant human-IL-1β, recombinant human-TNFα, and recombinant human-IFNγ. Recombinant human cytokines were used and purchased from Endogen (Woburn, MA); recombinant human IL-1β (specific activity-1 x $10^7$U/mg), recombinant human IFNγ(specific activity-3 x $10^7$U/mg) and TNF α(specific activity-≥2 x $10^7$U/mg) were added in 25ng, 125ng and 250ng quantities respectively. Both experimental and control groups were cultured in 10ml of supplemented Hams F-10 medium for 48 hours at 37 °C in 95% air/ 5% $CO_2$. Islet cultures were again washed twice and samples were taken for cellular insulin and DNA contents, functional viability and northern and western blot analysis.

**Characterization of neonatal porcine islets**

Prior to cytokine exposure, the cellular composition of NIC aggregates was determined by immunohistochemistry. The avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzadine as the chromagen. Sections (1 μm) were affixed to glass slides by heat, the plastic resin removed with sodium methoxide and counter stained with Harris's hemotoxylin for 2 min., then subsequently stained separately for the presence of insulin, glucagon and cytokeratin-7 (CK-7, ductal cell marker) positive cells. In each experiment, a minimum of 15 aggregates randomly selected from 3 to 4 different sections were examined. Primary antibodies (Dako, Carpinteria, CA) included guinea pig anti-porcine insulin (1:1000), rabbit anti-glucagon (1:100) and a mouse anti-human CK-7. Biotinylated secondary antibodies and the ABC-enzyme complexes were purchased from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 min. (room temperature), while secondary antibodies were applied for 20 min.

Following 48 hour culture, samples of both porcine NIC aggregates exposed to human cytokines and non-treated porcine NIC serving as controls were evaluated

using immunohistochemistry. The avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzadine as the chromagen. Sections (1 µm) were affixed to glass slides by heat, the plastic resin removed with sodium methoxide and counter stained with Harris's hemotoxylin for 2 min., then subsequently stained separately for the presence of insulin, as in above. Exposed samples and controls were compared for differences in morphology and levels of insulin staining.

Insulin content, from aliquots taken on day zero and following 48 hour cytokine exposure, was measured after extraction in 2mmol/ liter acetic acid containing 0.25% RIA grade BSA. Samples were sonicated in acetic acid, centrifuged (1500 RPM for 10 minute). Supernatant was collected and concentrated using a speed vacuum (Savant speed vacuum with refrigerated vapour trap, Model SC-110, Savant Instr. Inc. Holbrook, NY) to remove acetic acid. Samples were then reconstituted in 1ml phosphate buffered saline (160 mmol/l NaCl, 0.5% RIA grade BSA, and 10mmol/l $NaH_2PO_4$-$2H_2O$) and stored at 4 °C until assayed for insulin content by radioimmunoassay (RIA 100 kit, Pharmacia-Upjohn, Uppsala, Sweden).

For DNA content, aliquots were taken at day zero and following 48 hour cytokine exposure, cells were washed in citrate buffer (150 mmol/liter NaCl, 15mmol/liter citrate, 3mmol/liter EDTA, pH-7.4) and stored as cell pellets at –20$^{O}$C. Before being assayed, cell pellets were placed in 450µl of lysis buffer (10mmol/liter Tris, 1mmol/liter EDTA, 0.5% Triton X-100, 4 $^{O}$C, pH 7.5), sonicated, supplemented with 25µl of Proteinase K solution (8 mg/ml), vortexed, and incubated for 1 hour at 37 $^{O}$C. Aliquots of 25 and 50 µl were assayed in duplicate by diluting them in 1ml of DNA buffer (10 mmol/liter Tris, 1 mmol/liter EDTA, pH 7.5) and measuring fluorescence at 490 exc./ 515 em. nm after the addition of 1ml of Pico Green reagent (1/200 dilution with DNA buffer). Samples were run in parallel with and diluted in proportion to a seven point (0-400 ng/ml) standard curve, which was generated using calf thymus DNA.

Percent recovery was calculated for both insulin and DNA contents. Samples from both experimental and control groups were taken on day zero. This allowed for calculation of total DNA and insulin per aliquot loaded per non-treated tissue culture plate. Samples from day two were also taken and analyzed to reveal the amount of

DNA and insulin remaining after culture with or without cytokines. Percent recovery was calculated for both porcine and human islet preparations using the following formula:

$$\text{Percent Recovery} = \frac{\text{Amount of DNA/Insulin-Day two (ng)}}{\text{Amount of DNA/Insulin-Day zero (ng)}} \times 100\%$$

For assessment of in vitro functional viability, the secretory response to glucose of human islets and neonatal porcine islet cells was determined, following 48 hours cytokine exposure, using a static incubation assay (2). The cultured fractions were recovered from the petri dishes, washed, and aliquots of 50-100 aggregates of human islets and porcine NIC were incubated for 120 minutes in 1.5ml of Hams F-10 supplemented with 2mmol/liter L-glutamine, 0.5% RIA grade BSA and either 2.8mmol/liter glucose or 20 mmol/liter glucose. Tissue and medium were then separated by gravity sedimentation and assayed for their respective insulin contents. The insulin content of the medium was expressed as a percentage of the total content of aliquot (tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/liter glucose by that released at 2.8mmol/liter glucose.

### Northern blot analysis

The porcine insulin cDNA in PCR 2.1 TOPO (Invitrogen) vector was isolated and sequenced. The 252 bp porcine insulin insert was excised with Eco R1 restriction endonuclease (New England Biolabs, Inc., Beverly, MA). The insert hybridized only with insulin transcripts in northern blot analyses. The cDNA insert was separated on a 1.0% agarose gel, extracted and purified with GLASSMILK silica matrix (Bio 101, Inc., La Jolla, CA). Using a hexamer primer of random sequence, the $^{32}$P-labelled probe was synthesized from the insert cDNA as specified by the manufacturer (Random Primers Labeling System, Life Technologies, Burlington, Ontario). Total RNA was isolated from approximately 600-1000 porcine NIC aggregates from both control and experimental groups from several islet preparations using Trizol reagent (Life Technologies, Burlington, Ontario). Briefly, tissue was homogenized, and RNA isolated by phenol-chloroform extraction and precipitation with isopropanol.

For Northern blot analyses, 10µg of total RNA was separated by electrophoresis through an 1.5% denaturing formaldehyde agarose gel, transferred to nylon filters (Micron Separations, Inc., Westboro, Ma) and baked under vacuum at 80 $^{O}$C for 1 hour. Hybridization was conducted in a solution containing 50% formamide, 5% dextran sulfate, 1% SDS, 5X SSPE (0.75M NaCl, 50mM $NaH_2PO_4$, 5mMol EDTA), 5X Denhardt's solution (0.1% Ficoll, type 400, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin, Fraction V), 100µg/ml denatured, sonicated salmon sperm DNA and radiolabelled cDNA probe ($1 \times 10^6$ cpm/ml) at 42 $^{O}$C for 16 hr. The final wash was under stringent conditions, 0.1X saline sodium citrate (0.1X SSC: 15mM NaCl. 1.5mM sodium citrate) and 0.1% SDS, at 55 $^{O}$C for 30 min. Fuji Super RX film was exposed to filters with two intensifying screens at -80 $^{O}$C for autoradiography. After autoradiography, the filter was stained with methylene blue to control for RNA loading. The band intensities on autoradiograms and after methylene blue staining were measured by an image analyzer (ChemiImager v5.5, Alpha Innotech Corporation, San Leandro, CA) to determine the levels of RNA hybridization and RNA loading, respectively. Insulin mRNA levels were normalized to rRNA levels to take into account differences that may have occurred during RNA loading. Statistical evaluation was performed on normalized values. RNA ladder (New England Biolabs, Inc., Beverly, Ma) was subjected to electrophoresis in the same gel as a molecular weight marker.

### Western blot analysis

Total soluble proteins were isolated from both experimental and control porcine NIC aggregates. The cells were lysed for 30 min on ice in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% deoxychloride, 0.1% SDS, 1 mM EDTA, 5 mM NaF) containing a cocktail of proteinase inhibitors (1 mM PMSF, 1 mM DTT, protease inhibitors (10µl/ml P8340 Sigma Chemical Co., St. Louis, MO.)). The extracts were then centrifuged at 12000 rpm for 5 min at 4°C and the supernatant was collected as soluble protein. Protein concentration was determined by the method of Bradford, with BSA as the standard (24).

Thirty micrograms of protein were loaded and subjected to electrophoresis on a 10% SDS polyacrylamide gel. Seven separate protein preparations were used. The proteins were transferred to Immune-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA), blocked with 5% blotto in TBS for 1 hr at room temperature and then incubated with rabbit polyclonal anti-PDX1 (1:10000) in TBS-T for 1 hr at room temperature, followed by incubation with horseradish peroxidase-conjugated anti-rabbit (1:5000; Amersham Pharmacia Biotech, San Francisco, CA) secondary antibody in TBS-T for 30 min at room temperature. The proteins were detected by the Enhanced Chemiluminescence (ECL) Western blotting system (Amersham Pharmacia Biotech, San Francisco, CA) as described in the protocols from the supplier. The band intensities, before and after coomasie blue stain, were measured by an image analyzer (ChemiImager v5.5, Alpha Innotech Corporation, San Leandro, CA) to determine the levels of protein and protein loading respectively. PDX-1 levels were normalized to an arbitrary band that showed strong coomasie blue staining to take into account differences that may have occurred during protein loading. Statistical evaluation was performed on normalized values.

**Measurement of caspase 3 activity**

Caspase 3 activity was determined using a colorimetric assay with a DEVD-pNA substrate (BIOMOL Research Laboratories Inc., PA, USA). Cells ($5 \times 10^5 - 1 \times 10^6$) in 100 µl if ice cold lysis buffer (50 mM HEPES, 10mM $MgCl_2$ and 0.25% (v/v) Nonidet P-40) were incubated on ice for 10 minutes. Samples were centrifuged for one minute at 1000 x g at 4 $^{O}$C and the supernatants transferred to fresh tubes. The protein concentration of the supernatants was determined using a BCA kit (Pierce, Rockford, IL, USA). Samples (50µl containing ~50µg of total protein) were mixed with 50µl of 2X reaction buffer (100mM HEPES, pH 7.5, 20% sucrose, 10mM dithioreitol and 0.2% (w/v) CHAPS). A DEVD-pNA substrate was added to each sample to a final concentration of 200µM. Samples were then incubated at 37 $^{O}$C for 2 hours followed by absorbance measurements. A positive control was generated by choosing one sample of porcine neonatal islet cells and incubating them with 1µl of granzyme B (100µg/ml), a potent activator of caspase 3. This sample was then run in

the same manner as other samples. Sample caspase 3 activity were expressed as percent of positive control.

### Statistical analysis

Data are expressed as means ± SEM of n independent observations, statistical significance of differences was calculated with a two-tailed unpaired Student's t test.

## Results

### Effect of human cytokines on islet recovery and viability

DNA recovery was assessed and used as a measure of islet cell recovery. Exposure of porcine NIC to human cytokines for 48 hours had no effect on DNA recovery when compared with control porcine NIC not treated with human IL-1$\beta$, TNF$\alpha$ and IFN$\gamma$ (99.9% ± 13.88 vs. 123.8% ± 16.11, respectively- see Table 1). Recovery of cellular insulin content was also observed and taken before and after cytokine exposure. In contrast to DNA recovery, treatment of porcine NIC with human cytokines resulted in a significant loss of cellular insulin when compared to non-exposed controls (56.6% ± 8.13 vs. 104.4%±12.15 respectively, $p<0.01$). Human islets served as a control to ensure that our assay using IL-1$\beta$, IFN$\gamma$, and TNF$\alpha$ was effective. Human islets exposed to the cytokine cocktail for 48 hours had a mean DNA recovery of 68.6%±13.06 and a mean cellular insulin recovery of 34.82 ± 7.29%. Similarly, control islets not subjected to cytokine treatment had a mean DNA recovery of 76.45% ± 3.80% and 36.1% ± 9.72% for both DNA and Insulin respectively. There was no significant difference between insulin or DNA recovery results for human islets treated with cytokines and those serving as controls (Table 1). However, when human islets exposed to cytokines were examined for glucose stimulated insulin secretion there was an adverse effect seen. Stimulation index (SI) is a measure which compares the level of islet cell response at high glucose concentration to that at basal levels. It reveals the ability of islet cell preparations to mount a response to a glucose challenge. Pro-inflammatory cytokines did affect the ability of human islets to respond to a glucose challenge. There was a significant difference in response to low glucose levels noticed in human islets exposed to

cytokines when compared to controls (5.56% ± 0.79 vs. 9.907% ± 1.78 respectively, $p<0.05$). A significant decrease in the response to a high glucose challenge was also noticed in treated human islets (7.26% ± 1.90 versus 24.7% ± 6.60 for control samples, $p<0.05$). A second indicator of a poor response is the low stimulation index of 1.07 ± 0.24 observed in treated human islet cells. This value is statistically different ($p<0.05$) than the stimulation index for control human islets (2.46 ± 0.44) which, unlike those treated with cytokines, showed a clear secretory response.

The secretory activity of porcine NIC was tested by comparing the percent of cellular insulin release at low glucose (2.8mM) and high glucose (20.0mM). Cytokine treatment did not effect the secretory response of porcine NIC as there was no significant difference in the stimulation indices of treated and non-treated cultures (2.63 ± 0.29 for exposed vs. 2.71 ± 0.22 for control). Although the SI values remained similar indicating a similar level of response, there was an increase noticed in both the mean basal and high glucose response values when compared to control preparations. A significant difference in basal glucose response (8.30% ± 1.28- experimental vs. 5.11% ± 0.77- controls, $p<0.05$) and high glucose response (19.85% ± 2.56-exposed vs. 12.90% ± 1.82-non exposed, $p<0.05$) was observed in exposed porcine NIC when compared to non-exposed porcine controls.

Immunohistological examination confirmed that porcine preparations prior to cytokine exposure consisted mainly of ductal cells (54.5% ± 6.38%) with the presence of 20% ± 1.24% insulin-containing and 19.5% ± 1.53% glucagon-containing cells scattered randomly throughout the aggregate. This is similar to historical data previous studies performed in our lab. Following 48 hour culture, samples of porcine neonatal islet cells either exposed to human cytokines or non-treated controls were fixed on slides and stained immunohistochemically for insulin (see Figure 1). Slides were also examined for proper morphology. There was no observable difference between treated and non-treated porcine neonatal islet cells as both appear normal and show insulin positive cells.

### Effects of human cytokine exposure on caspase three activity

In order to assess if apoptosis was occurring due to cytokine exposure I performed a caspase 3 assay. Caspase 3, is a protein associated with apoptosis. Apoptosis is a result of specific proteolytic events mediated by caspases. Caspase 3 is an apoptosis effector enzyme, that when activated participates in carrying out the properties of apoptosis such as DNA fragmentation and fragmentation of the nuclear membrane. Caspase 3 activity was determined by colorimetric assay with a DEVD-pNA substrate. Granzyme B, a potent activator of caspase 3, was added to one porcine NIC sample and caspase 3 activity was measured and used as a positive control. I expressed all values as a percent of positive control (see Fig. 3). Exposure to cytokines did not produce a statistically significant difference in caspase 3 activity when compared to control cultures ( 54.8% ± 14.2 for exposed vs. 47.4% ± 10.3 in controls, n = 11 for both treated samples and controls). Cytokines do not upregulate caspase 3 activity in porcine NIC when compared to porcine islets cultured without cytokines.

### Western blot analysis for PDX-1 protein expression

PDX-1, or pancreas duodenal homeobox-1 protein, is a transcription factor crucial for the development of progenitor cells into insulin positive beta cells. PDX-1 is also crucial in islet gene expression and may be required for expression of the insulin gene (25). I generated a western blot of protein from islet cells exposed to cytokines (n=7) and from non-treated porcine islet cell cultures (n=7) to test if differential PDX-1 expression may be causing the observed marked decrease in total insulin content (See Fig. 2A). Expression of this transcription factor was observed in both experimental and control preparations. Our results show no significant difference in the PDX-1 expression of cytokine exposed and non-exposed porcine islets, as can be seen in the normalized intensity values of the PDX-1 protein on our western blot (PDX-1 protein intensity with cytokines- 1.107 ± .111 vs. PDX-1 without cytokines 1.074 ± 0.089).

### Northern blot analysis for insulin mRNA levels

I also examined the expression of the insulin gene by probing a northern blot of total cellular RNA of several experimental (n=3) and control preparations (n=3) for insulin mRNA (see Fig 2B). It was believed that cytokines may be exerting an effect on the expression of insulin mRNA resulting in a drop in insulin synthesis. Again, I observed the presence of the insulin mRNA in both experimental and control groups. Moreover, there also was no significant difference between the normalized intensity of the insulin gene mRNA levels of both treated and non-treated groups on our northern blot (insulin mRNA intensity with cytokines- 1.949 ± 0.091 vs. insulin mRNA intensity without cytokines- 1.745 ± 0.084).

Table 1. Effect of human cytokines on neonatal porcine islet survival and functional viability.

| Condition | % Recovery (n) |  | Glucose Stimulated Insulin Secretion (n) |  |  |
|---|---|---|---|---|---|
|  | **Insulin** | **DNA** | **2.8 mM** | **20 mM** | **SI** |
| ***Pig Islets*** |  |  |  |  |  |
| **+ Cyto** | 56.6 ± $8.13^{\ddagger}$ (11) | 99.9 ± 13.88 (11) | 8.30 ± 1.28* (12) | 19.85 ± 2.56* (12) | 2.63 ± 0.29 (12) |
| **- Cyto** | 104.4 ± 12.51 (11) | 123.8 ± 16.11 (11) | 5.11 ± 0.77 (12) | 12.90 ± 1.85 (12) | 2.71 ± 0.22 (12) |
| ***Human Islets*** |  |  |  |  |  |
| **+ Cyto** | 34.82 ± 7.29 (6) | 68.6 ± 13.06 (3) | 5.56 ± 0.79* (6) | 7.26 ± 1.90* (6) | 1.07 ± 0.24* (6) |
| **- Cyto** | 36.1 ± 9.72 (6) | 85.8 ± 9.73 (3) | 9.91 ± 1.78 (6) | 24.70 ± 6.20 (6) | 2.46 ± 0.44 (6) |

Values are expressed as means ± SEM. Number of porcine or human islet preparations for each experiment are listed in brackets (n). Statistical significance of differences was calculated using a Student's unpaired t-test. *P<0.05, ‡ P<0.01 vs. islet preparations not exposed to cytokines.

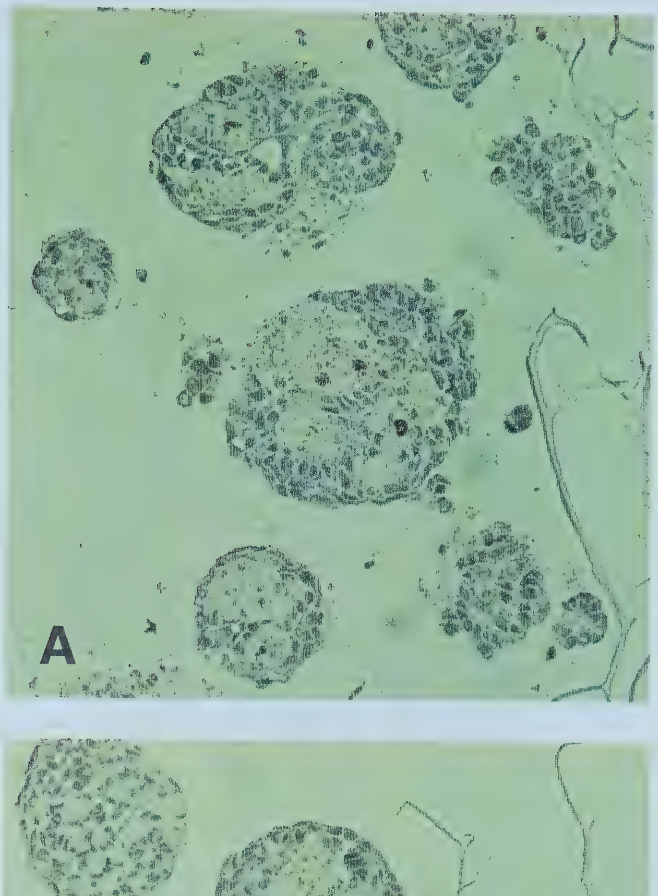

**Figure 1:** Light micrographs of neonatal porcine islet cells (A) exposed to human cytokines, (B) control islets not treated with human cytokines. Sections were immunohistochemically stained for insulin (brown).

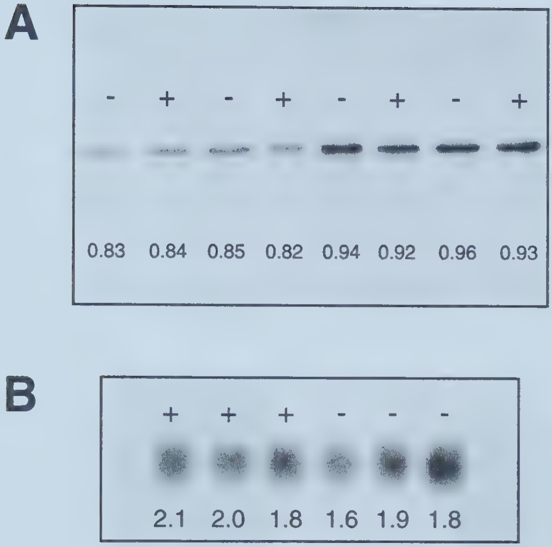

**Figure 2:** (**A**) Expression of PDX-1 protein on a western blot of four independent neonatal porcine islet preparations either exposed (+) or not exposed (-) to human IL-1β, IFNγ and TNFα. There was no significant difference in PDX-1 protein levels between exposed and non-exposed islet cells. (**B**) A northern blot showing expression of porcine insulin mRNA in three independent neonatal porcine islet preparations either exposed or not exposed to human cytokines. There was no significant difference in porcine insulin mRNA expression between exposed and non-exposed islet cells. Numerical values in both figures represent normalized values of the band intensity of PDX-1 and insulin mRNA respectively. In A, the mean±SEM values of intensity for the PDX-1 bands were 1.107±0.11 and 1.074±0.089 for exposed (+) and non-exposed (-) islet cells respectively. In B, the mean±SEM values of intensity for the mRNA bands were 1.949±0.091 and 1.754±0.084 for exposed (+) and non-exposed (-) islet cells respectively.

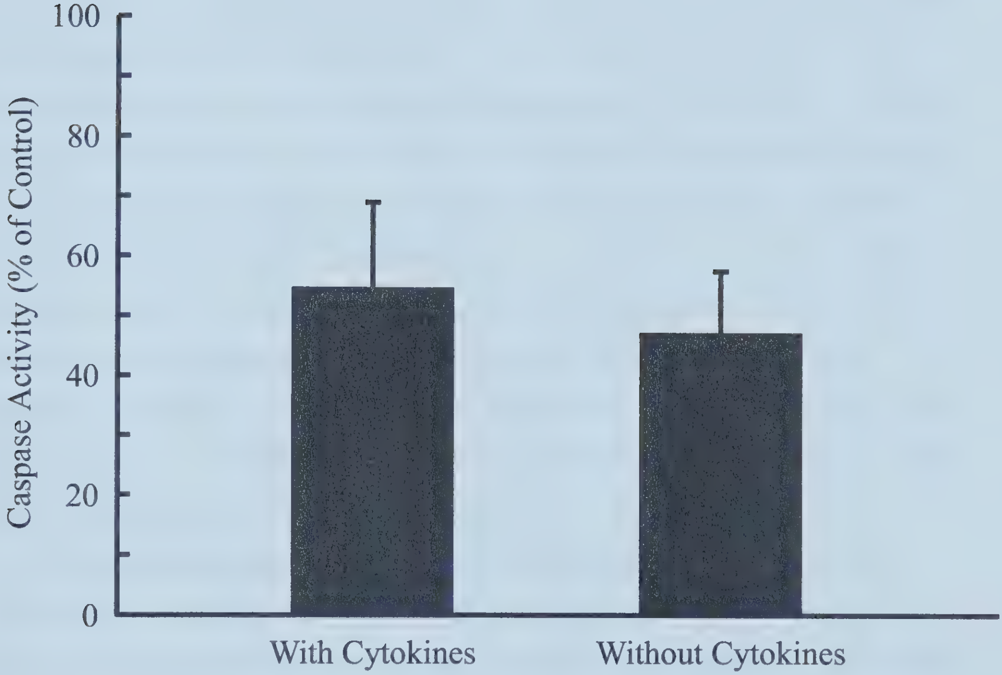

**Figure 3:** Average caspase 3 activity (n=11), expressed as a percent of positive control activity, of neonatal porcine islets. The data represents the average ± SEM for caspase 3 activity of porcine neonatal islet cells exposed to human cytokines (54.8% ± 14.2) and control neonatal porcine islet cells not exposed (47.4% ± 10.3).

## Discussion

Neonatal porcine islet cells represent an attractive tissue source for islet transplantation since they are readily available and have the capacity to differentiate and proliferate (2). Neonatal porcine islet cells however evoke a number of immunological responses from the immune system of human recipients. Our lab has previously shown that encapsulated porcine NIC may circumvent complement mediated cell lysis and that cytotoxic human T cells may not respond to porcine NIC. There is a lack of research looking into the effect of human cytokines on neonatal porcine cells. Previous studies have shown that human cytokines are able to inhibit islet function and cause cell death in rodent islets (16-18). Human pro-inflammatory cytokines such as IL-1β, IFNγ, and TNFα have also been previously shown to be cytotoxic to human islets (20). Individually, it was discovered, that none of these cytokines was able to mediate a significant effect on human islets. When the three cytokines were combined, however, cell dysfunction and cell death occurred. Therefore, I chose to use all three cytokines in our assay since it is likely that all three cytokines will be encountered post-transplant. In this study, human islets served as a control to ensure the cytokine exposure was effective.

One purpose of our study was to examine the functional effects of these cytokines on porcine NIC. Total cellular insulin levels decreased significantly in porcine islets exposed to human cytokines when compared to controls. The cytokines did not affect the insulin secretory response of the porcine NIC as both treated and non-treated controls yielded similar stimulation indices (2.63 ± 0.29 vs. 2.71 ± 0.22 respectively) following exposure to 2.8 and 20.0 mM glucose. In contrast, human islets were unable to up-regulate their secretion of insulin in response to a glucose challenge when treated with cytokines. The increase in both the mean basal and high glucose responses of exposed neonatal porcine islets can be explained. These values are expressed as a percentage of total cellular insulin, and total cellular insulin in this case was significantly reduced causing an increase in both glucose response values.

I was interested as to why total cellular insulin decreased by 48% after cytokine exposure. Cell death could be one reason for this observation. In contrast

to this, I saw no significant loss in DNA between exposed and non-exposed cultures suggesting that the recombinant human cytokines did not cause cell death. Moreover, I also performed an activated caspase 3 assay on several experimental and control islet preparations. Caspase 3 is a protein closely associated with pro-apoptotic cascades. There was no significant difference noted in the presence of activated caspase 3 between treated and non-treated islet cell cultures thus cell death does not appear to be responsible for the cytokine induced reduction in cellular insulin.

Cytokines may cause a decrease in the total cellular insulin of porcine NIC by affecting expression of the insulin gene. I hypothesized that cytokines decreased protein levels of PDX-1, a transcription factor crucial for proper expression of the insulin gene. I saw no observable difference in PDX-1 protein expression between porcine NIC treated with cytokine and controls. Therefore, the reduction of cellular insulin after cytokine exposure was not related to lower PDX-1 expression. Ling et al, however, showed earlier that PDX-1 expression was down regulated in purified rat beta cell preparations when exposed to recombinant human IL-1 beta (26). This study also revealed that the glucose transporter 2 (GLUT 2) and proinsulin convertase 2 (PC2) were also down regulated in rat beta cells exposed to human cytokines. The decreased expression of PDX-1, GLUT 2 and PC2 may alter beta cell phenotype making the cells less responsive

Another explanation is that human cytokines may lower total cellular insulin by altering the level of insulin mRNA. I developed a northern blot of total RNA from exposed and non-treated NIC. This blot was then probed for porcine insulin mRNA levels in experimental and control groups. Again, cytokines do not seem to be effecting cellular insulin levels by decreasing the expression of the islet insulin gene.

Another possibility is that the pro-inflammatory cytokines are inducing degranulation of porcine islet beta cells. A ratio of insulin recovery to DNA recovery can be compared to control groups and used to suggest cellular degranulation. If degranulation is occurring, then I would expect to see a lower ratio of insulin to DNA recovery when compared to non- exposed control islets. In our exposed islet cell preparations I saw no significant decrease in percent DNA recovery, however, a large drop in total cellular insulin was observed. The ratio of percent insulin recovery to

percent DNA recovery is 0.567 in treated porcine groups, whereas the ratio in control porcine NIC was 0.840. This suggests that the total insulin drop in experimental groups may be due to cytokine induced degranulation of porcine beta cells.

I have not discovered the reason for degranulation of insulin positive cells treated with cytokines. Our studies suggest that the cytokines do not effect the expression of the insulin gene or its transcription factor, PDX-1. Another possibility is that cytokines lower cellular insulin by effecting insulin synthesis. Insulin is expressed as an inactive 9000kd-proinsulin molecule. It is processed by several proinsulin convertases, which result in active insulin production. A study by Hostens et al has shown that human pro-inflammatory cytokines can downregulate parts of the insulin converting machinery, specifically PC1 and PC2, in human islets resulting in a disproportionate accumulation of proinsulin (27). It is possible that human cytokines are causing similar effects in porcine islet cells. Future studies should now test this hypothesis.

It should be taken into account that this study was conducted in vitro. If porcine islets are ever used as a tissue source for clinical islet transplantation, they will be exposed to human pro-inflammatory cytokines in a different environment and possibly in a more direct fashion. Cytokine products from invading lymphoid cells may be delivered in a specific, direct fashion that is not mimicked by in vitro models (15). In his review, Pipeleers et al list several disadvantages of in vitro models: such as they fail to take into account fluctuating variables that occur in vivo, amount of tissue in preparations is often insufficient to allow for molecular analysis of processes occurring during cell death, and tissue is not available from diabetic phases in humans (28). Thus, we should be cautious not to extrapolate data from our in vitro studies and use it to evaluate the transplant potential of porcine neonatal islet cells.

I set out to evaluate whether the human cytokines-IL-1β, IFNγ, and TNFα would effect the function of porcine NIC. Unlike human islets, the porcine islet insulin secretory response or cell survival was not effected by these human cytokines. I observed that these cytokines may cause the degranulation of porcine insulin positive cells resulting in reduced total cellular insulin of treated cultures. Human

cytokines do not effect the expression of the porcine insulin gene or its transcription factor, PDX-1.

Due to the limited availability of human pancreases, porcine islets may be the best option for pancreatic islet cell transplantation because they are readily available and the similarities between human and porcine islet physiology. Studies have shown that human CD8+ T cells are unable to respond to porcine NIC(13), moreover, our lab has discovered a possible solution to hyperacute rejection using microencapsulation (12). The next immunological challenge is presented by pro-inflammatory cytokines that may be released by infiltrating leukocytes. Our study suggests, contrary to our hypothesis, that human pro-inflammatory cytokines are not directly cytotoxic to porcine NIC. Also contrasting with our hypothesis, I saw no adverse effects on the ability of exposed neonatal porcine islet cells to respond to a glucose challenge. Further studies should, however, elucidate why cytokines reduce cellular insulin levels, and also test if this affect alters long term function.

## References

1. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343: 230.

2. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119.

3. Ricordi C, Socci C, Davalli AM, et al. - Isolation of the elusive pig islet. - Surgery 1990 Jun;107(6):688-94. 688.

4. Marchetti P, Giannarelli R, Cosimi S, et al. - Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. - Diabetes 1995 Apr;44(4):375-81. 375.

5. Wright JR, Polvi S, MacLean H. - Experimental transplantation with principal islets of teleost fish (Brockmann bodies). Long-term function of tilapia islet tissue in diabetic nude mice. - Diabetes 1992 Dec;41(12):1528-32. 1528.

6. Ferber S, BeltrandelRio H, Johnson JH, et al. - GLUT-2 gene transfer into insulinoma cells confers both low and high affinity glucose-stimulated insulin release. Relationship to glucokinase activity. - J Biol Chem 1994 Apr 15;269(15):11523-9. 11523.

7. Knaack D, Fiore DM, Surana M, et al. - Clonal insulinoma cell line that stably maintains correct glucose responsiveness. - Diabetes 1994 Dec;43(12):1413-7. 1413.

8. Kover K, Moore WV. - Development of a method for isolation of islets from human fetal pancreas. - Diabetes 1989 Jul;38(7):917-24. 917.

9. Bonner-Weir S, Taneja M, Weir GC, et al. In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 2000; 97: 7999.

10. Bradley JA. - Overcoming the immunological barriers to xenotransplantation. - Transplantation 1999 Jul 15;68(1):9-11. 9.

11. Rayat GR, Rajotte RV, Elliott JF, Korbutt GS. - Expression of Gal alpha(1,3)gal on neonatal porcine islet beta-cells and susceptibility to human antibody/complement lysis. - Diabetes 1998 Sep;47(9):1406-11. 1406.

12. Rayat GR, Rajotte RV, Ao Z, Korbutt GS. Microencapsulation of neonatal porcine islets: protection from human antibody/complement-mediated

cytolysis in vitro and long-term reversal of diabetes in nude mice. Transplantation 2000; 69: 1084.

13. Murray AG, Nelson RC, Rayat GR, Elliott JF, Korbutt GS. - Neonatal porcine islet cells induce human CD4+, but not CD8+, lymphocyte proliferation and resist cell-mediated cytolytic injury in vitro. - Diabetes 1999 Sep;48(9):1713-9. 1713.

14. Bach JF. - Insulin-dependent diabetes mellitus as an autoimmune disease. - Endocr Rev 1994 Aug;15(4):516-42. 516.

15. Rabinovitch A, Suarez-Pinzon WL. - Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. - Biochem Pharmacol 1998 Apr 15;55(8):1139-49. 1139.

16. Mandrup-Poulsen T, Bendtzen K, Nerup J, Egeberg J, Nielsen JH. - Mechanisms of pancreatic islet cell destruction. Dose-dependent cytotoxic effect of soluble blood mononuclear cell mediators on isolated islets of Langerhans. - Allergy 1986 May;41(4):250-9. 250.

17. Sandler S, Andersson A, Hellerstrom C. - Inhibitory effects of interleukin 1 on insulin secretion, insulin biosynthesis, and oxidative metabolism of isolated rat pancreatic islets. - Endocrinology 1987 Oct;121(4):1424-31. 1424.

18. Southern C, Schulster D, Green IC. - Inhibition of insulin secretion by interleukin-1 beta and tumour necrosis factor-alpha via an L-arginine-dependent nitric oxide generating mechanism. - FEBS Lett 1990 Dec 10;276(1-2):42-4. 42.

19. Scarim AL, Heitmeier MR, Corbett JA. - Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1beta. - Endocrinology 1997 Dec;138(12):5301-7. 5301.

20. Rabinovitch A, Sumoski W, Rajotte RV, Warnock GL. - Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. - J Clin Endocrinol Metab 1990 Jul;71(1):152-6. 152.

21. Corbett JA, Sweetland MA, Wang JL, Lancaster JR, McDaniel ML. - Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. - Proc Natl Acad Sci U S A 1993 Mar 1;90(5):1731-5. 1731.

22. Warnock GL, Ellis D, Rajotte RV, Dawidson I, Baekkeskov S, Egebjerg J. - Studies of the isolation and viability of human islets of Langerhans. - Transplantation 1988 May;45(5):957-63. 957.

23. Korbutt GS, Rayat GR, Ezekowitz J, Rajotte RV. - Cryopreservation of rat pancreatic islets: effect of ethylene glycol on islet function and cellular composition. - Transplantation 1997 Oct 15;64(7):1065-70. 1065.

24. Bradford MM. - A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal Biochem 1976 May 7;72:248-54. 248.

25. Peshavaria M., Stein R. PDX-1: An Activator of Genes Involved in Pancreatic Development and Islet Gene Expression. In: Pancreatic Growth and Regeneration, Sarvetnick Nora, ed. La Jolla, CA, Karger Landes Systems, 1997; 96

26. Ling Z, Van De Casteele M, Eizirik DL, Pipeleers DG. Interleukin-1beta-induced alteration in a beta-cell phenotype can reduce cellular sensitivity to conditions that cause necrosis but not to cytokine-induced apoptosis. Diabetes 2000; 49: 340.

27. Hostens K, Pavlovic D, Zambre Y, et al. - Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release. - J Clin Invest 1999 Jul;104(1):67-72. 67.

28. Pipeleers D, Hoorens A, Marichal-Pipeleers M, Van De Casteele M, Bouwens L, Ling Z. - Role of pancreatic beta-cells in the process of beta-cell death. - Diabetes 2001 Feb;50 Suppl 1:S52-7. S52.

# General Discussion and Conclusion

Insulin dependent diabetes mellitus is a disease resulting from an autoimmune destruction of insulin producing beta cells by invading immune leukocytes. Cytokines, soluble immune messengers, are thought to be an important role player in the inhibition and eventual destruction of islet cells in diabetes (1,2). Specifically, Th1 associated cytokines IL-1β, IFNγ and TNFα are thought to inhibit insulin synthesis and function as well as induce necrosis and apoptosis of islet beta cells. Initially it was discovered that pro-inflammatory cytokines inhibit function and viability of rat islets (3). A study by Southern et al examined whether inducible nitric oxide synthase was responsible for the effects of cytokines. Inducible nitric oxide synthase (iNOS) increases nitric oxide levels in cells, a product with negative effects on cellular respiration and that may mediate DNA damage (4). Macrophages and ductal cells may also be induced to produce nitric oxide by these cytokines leading to further damage to islet cells (5-7).

Similar work on human islets was carried out several years later by Rabinovitch et al. In his study, he showed that the cytokines, IL-1β, IFNγ and TNFα, are cytotoxic to human insulin and glucagon positive cells (8). Several other studies discovered that these cytokines can induce DNA damage (9), down regulate insulin-processing (10), and increase iNOS and nitric oxide levels (11). However, it should be noted that human islets are more resistant to cytokine induced damage than rodent islets and iNOS alone may not be sufficient to damage human islets (12).

Islet transplantation is the most likely solution to replacing pancreatic endocrine tissue since islets can be cryopreserved and stored, manipulated in vitro and have been shown to restore euglycemia in patients with type 1 diabetes. However, there is a limited supply of human cadaveric pancreas tissue. Therefore, it is important to explore other animal sources of islet tissues as possible candidates for human islet transplantation. Porcine islets have been studied extensively (13,14) and may provide the best alternative tissue source since they are inexpensive, ethically acceptable and have similar physiology to humans. Neonatal porcine islets present an attractive source of insulin producing tissue for clinical transplantation due to their

unlimited supply and inherent ability to proliferate and differentiate (13). It is important to understand the response of the human immune system to porcine islet xenografts if they are to be used as candidates for transplantation. Our lab has researched hyperacute rejection and T cell mediated responses to neonatal porcine islets (15,16). Little attention, though, has been paid to address the effects of pro-inflammatory cytokines on neonatal porcine islets. Since, it appears that these immune mediators may be involved in diabetic autoimmune responses it is crucial to understand how they may effect neonatal porcine islet tissue.

I performed a study in which neonatal porcine islets were subjected to human IL-1β, IFNγ and TNFα in vitro for 48 hours. I then evaluated cell survival and any functional effects of human cytokines on neonatal porcine islets. I tested for glucose stimulated insulin response, viability and searched for signs of cell death. I hypothesized that exposure of neonatal porcine islet cells to the proinflammatory human cytokines, IL-1β, IFNγ and TNFα, would result in cell death and reduced glucose stimulated insulin secretion. However, I found that although human pro-inflammatory cytokines were not able to effect the survival or insulin secretory response of neonatal porcine islets, they were able to significantly reduce the levels of cellular insulin. Furthermore, there was no evidence that cytokines altered the expression of the insulin gene as PDX-1 protein and insulin mRNA levels were not affected by cytokine treatment.

Our research is not complete enough to allow us to confidently speculate on the transplant potential of porcine neonatal islet cells. I discovered that neonatal islets exposed to cytokines had the ability to respond to a glucose challenge. The similar secretory stimulation index suggests that human IL-1β, IFNγ and TNFα do not damage the secretory response of neonatal islet cells. However, I did observe a significant loss of total cellular insulin of porcine cultures treated with human pro-inflammatory cytokines. This reduction in total cellular insulin may affect the long-term function of porcine islet grafts in vivo. Transplanted grafts will be under great demand to supply insulin in a regulated manner. Cytokines, by affecting total graft insulin content may prevent porcine grafts from achieving long term euglycemia. An interesting study by Korsgren et al used a clever in vivo model to look into the effect

of discordant xenograft rejection on syngeneic islet function (17). Xenogeneic fetal pig islets were co-transplanted with syngeneic mouse islets into a mouse model. Syngeneic mouse islets exposed to the xenograft rejection mechanism were functionally impaired and did not attain complete functional recovery. This study reveals, indirectly, that islets exposed to the mechanism of xenograft rejection may be functionally inhibited. Our study is the first step in discovering the effects of human cytokines on porcine tissue. However, if we are to fully understand the effect of human cytokines on the transplant potential of porcine neonatal islet cells than there are clearly many more steps to be taken.

Future studies are clearly required in order to fully understand the mechanisms by which human cytokines alter insulin content of neonatal porcine islet cells. First, the focus of future research should be to establish whether iNOS and nitric oxide are both being expressed and playing a role in cytokine exposed porcine NIC. Nitric oxide synthase is the main mediator of damage in rodent islets exposed to human recombinant cytokines (18-20); cytokines may also induce iNOS expression in human islets (11). Although there is no consensus in the degree to which nitric oxide mediates the damage caused by cytokines, it is known that it affects mitochondrial function, and cellular respiration and therefore could be involved in the reduction of cellular insulin levels (4). Northern and western blots could be used to first establish its presence. Nitrite and nitrate are both a byproduct of nitric oxide, by performing assays aimed at revealing the nitrite and nitrate levels of neonatal porcine islet cells one can also indirectly probe for iNOS. These future studies should assess for cellular damage mediated by iNOS and nitric oxide. Nitric oxide has been shown to induce DNA damage in both rodent and human islets. If iNOS is indeed being expressed in cytokine exposed porcine NIC than future research should examine these cells for signs of DNA damage.

It is unclear in rodent and human islets whether endogenous nitric oxide produced by islets subjected to IL-1β is sufficient to mediate islet cell death. Several labs have shown a role for macrophages in islet cell damage (5,6). Macrophages, when activated, can secrete cytokines (IL-1β) and nitric oxide which could both cause damage to transplanted islet cells. Macrophages have been shown to induce DNA

damage in rodent islets in vitro. Another study by Pavlovic et al has shown that human ductal cells may produce nitric oxide in response to IL-1β, IFNγ and TNFα (7). Neonatal porcine islet preparations have a large number of (~50%) CK 7 positive ductal cells. These cells may be contributing to the reduced islet cellular insulin by producing nitric oxide. If iNOS is found to play a role in the reduced cellular insulin content of treated porcine NIC, future studies must examine the role played by resident macrophages and ductal cells since both can produce nitric oxide in response to pro-inflammatory cytokines.

A study by Hostens et al showed that human pro-inflammatory cytokines produced a decrease in cellular insulin content, while total insulin (i.e. cell content plus that released into culture media) remained normal (10). It seemed that more insulin was being released into the media thus reducing total cellular insulin content. The cytokines also caused an increase in media proinsulin levels while cellular levels remained similar to those in controls. This effect was caused irrespective of nitric oxide formation. Proinsulin synthesis remained normal in cytokine induced islets, but conversion of proinsulin was markedly decreased. The authors showed a decrease in PC1 and PC2, the enzymes responsible for this conversion, thus cytokines may not alter cellular insulin by cytotoxic methods. The drop may simply occur due to changes in the proinsulin converting mechanism. Ling et al also showed that IL-1 was also able to reduce PC2 expression in purified rat islets. Our research on porcine islets showed a similar drop in total cellular insulin. Future studies should examine whether cytokine exposure to porcine NIC causes similar changes in the insulin processing. It is possible that these cells may also have elevated proinsulin that is not efficiently being converted to the secretory form of insulin.

A study by Scarim et al examined the ability of rodent islets to recover from cytokine treatment in vitro (19). Islets exposed for 24 hours and then washed and treated with an iNOS inhibitor were able to regain function and response to a glucose challenge. In contrast, rat islets subjected to a 36 or 48 hour cytokine treatment were unable to regain function even with the administering of an iNOS inhibitor. Future work on porcine islets should look intensely at the ability of these cells to recover

from differing lengths of cytokine culture. Second, examination of the ability of iNOS inhibitors to prevent cytokine-induced damage to porcine NIC should be performed. At this point, I am unsure if iNOS is actually playing a role in the reduction of cellular insulin content. If, however, iNOS is found important in later studies, the ability of these enzyme inhibitors is crucial since they may represent a strategy to overcome this post-transplant.

I performed some preliminary recovery studies on neonatal porcine islet cells. In our experiments, neonatal porcine islets were exposed to human IL-1β, IFNγ and TNFα for 48 hours as previously described. Non-exposed neonatal porcine islets served as controls (n=3 for both experimental and control groups). After two days of culture, experimental and control groups were washed twice, and representative aliquots for insulin recovery were taken. Exposed and control groups were then cultured for another 48 hours in supplemented Hams F-10 media. At the end of this culture, I again took representative samples for insulin recovery. Control cultures had a 72.3%±6.0 recovery on day two and a 70.9%±16.1 recovery on day four when compared to sample values from day zero. In contrast, exposed islets had a 38.2%±4.9 recovery on day two and a 39.0%±4.7 recovery on day four when compared to day zero values demonstrating that loss of cellular insulin content did not decrease further after cytokine exposure. This observation suggests that the initial reduction of cellular insulin content by cytokine exposure is transient. Furthermore, it is unlikely that exposed neonatal porcine islets will be able to fully recover in vitro, however, in vivo these islets might be able to increase their cellular insulin levels.

I repeated this experiment with a few modifications. After two day culture with or without cytokines, I performed recovery studies with the addition of exendin-4. Exendin-4 is a 39 amino acid peptide, from Gila lizards, that is 53% homologous to GLP-1 (21). GLP-1 is an incretin usually released in the gut to increase glucose stimulated insulin secretion. I hypothesized that exendin-4 may increase the ability of exposed islets to recover. However, the addition of exendin-4 actually reduced the total cellular insulin content of exposed and control islets on day four when compared to our previous study (35.03%±2.58 vs. 58.81±1.06 respectively, n=3). Exendin-4

reportedly increases both insulin expression and secretion (22). It is possible that exendin-4 is further degranulating these cells by increasing insulin secretion. It should be noted that Scarim et al performed their recovery studies with the addition of iNOS inhibitors. If iNOS is playing a role in the insulin drop noticed in exposed porcine neonatal islet cells than the addition of these inhibitors may have made a difference.

Last, it is important for future studies to evaluate the effect human IL-1β, IFNγ and TNFα on the ability of neonatal porcine islet cells to mature, develop and proliferate (13). As stated earlier, the reason neonatal porcine islet tissue is so attractive is because of its stability in culture and this ability to proliferate into new endocrine tissue. An interesting study would be one in which porcine NIC were exposed to IL-1β, IFNγ and TNFα, then monitored for development of new insulin and glucagon positive tissue.

Our study was done in vitro and therefore has several drawbacks, since it fails to take into account fluctuating variables that occur in vivo. It is difficult to design in vivo studies that monitor the effects of human cytokines on the ability of porcine islets to cure diabetes. I already discussed the Korsgren et al in vivo model. It fails to directly look at the ability of exposed xenogeneic islets to treat diabetes. One possibility involves the pre-exposure of porcine NIC to cytokines, followed by a wash and transplant into diabetic SCID mice. This would allow us to first see if these exposed islets can cure diabetes in lab animals, and I could also compare their performance to control porcine islets. Although this is not a direct in vivo study it does, however, provide us with some valuable information on the response of porcine tissue.

In summation, porcine islets present an attractive alternative to human islet tissue for islet cell transplantation. In order to make use of this tissue source we must first understand how the human immune system will respond to its presence. Extensive work has been performed on the antibody-complement mediated rejection of xenografts, and the response of human T cells to xenogeneic tissue. Yet, human

Th1 cytokines, which are thought to be important mediators of the autoimmune assault causing diabetes, have not been studied for possible effects on porcine islet tissue. Our study is the first to evaluate the effects of IL-1β, IFNγ and TNFα on neonatal pig tissue. I provided a glimpse of the effects these molecules can exert on porcine insulin positive cells. Future studies should be done to elucidate the mechanisms and pathways that are causing the effects seen in our study. Attention should be paid to mediators such as iNOS that has been shown historically to be a mediator of human cytokine induced damage. I must also examine the insulin processing machinery since changes to it may produce the drop in cellular insulin I observed. It is important for following work to focus on the causes of the alterations in porcine NIC that I noticed after exposure to human pro-inflammatory cytokines. It is only after we understand the effects of these mediators and the mechanisms in which they are produced that we can begin to suggest solutions, and fully evaluate the transplant potential of neonatal porcine islets.

## References

1. Rabinovitch A. - Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? - Diabetes 1994 May;43(5):613-21. 613.

2. Rabinovitch A, Suarez-Pinzon WL. - Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. - Biochem Pharmacol 1998 Apr 15;55(8):1139-49. 1139.

3. Mandrup-Poulsen T, Bendtzen K, Nerup J, Egeberg J, Nielsen JH. - Mechanisms of pancreatic islet cell destruction. Dose-dependent cytotoxic effect of soluble blood mononuclear cell mediators on isolated islets of Langerhans. - Allergy 1986 May;41(4):250-9. 250.

4. Eizirik DL, Flodstrom M, Karlsen AE, Welsh N. - The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. - Diabetologia 1996 Aug;39(8):875-90. 875.

5. Arnush M, Scarim AL, Heitmeier MR, Kelly CB, Corbett JA. - Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. - J Immunol 1998 Mar 15;160(6):2684-91. 2684.

6. Fehsel K, Jalowy A, Qi S, Burkart V, Hartmann B, Kolb H. - Islet cell DNA is a target of inflammatory attack by nitric oxide. - Diabetes 1993 Mar;42(3):496-500. 496.

7. Pavlovic D, Chen MC, Bouwens L, Eizirik DL, Pipeleers D. - Contribution of ductal cells to cytokine responses by human pancreatic islets. - Diabetes 1999 Jan;48(1):29-33. 29.

8. Rabinovitch A, Sumoski W, Rajotte RV, Warnock GL. - Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. - J Clin Endocrinol Metab 1990 Jul;71(1):152-6. 152.

9. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL. - Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. - Endocrinology 1997 Jun;138(6):2610-4. 2610.

10. Hostens K, Pavlovic D, Zambre Y, et al. - Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release. - J Clin Invest 1999 Jul;104(1):67-72. 67.

11. Corbett JA, Sweetland MA, Wang JL, Lancaster JR, McDaniel ML. - Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. - Proc Natl Acad Sci U S A 1993 Mar 1;90(5):1731-5. 1731.

12. Eizirik DL, Pipeleers DG, Ling Z, Welsh N, Hellerstrom C, Andersson A. - Major species differences between humans and rodents in the susceptibility to pancreatic beta-cell injury. - Proc Natl Acad Sci U S A 1994 Sep 27;91(20):9253-6. 9253.

13. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119.

14. Ricordi C, Socci C, Davalli AM, et al. - Isolation of the elusive pig islet. - Surgery 1990 Jun;107(6):688-94. 688.

15. Rayat GR, Rajotte RV, Ao Z, Korbutt GS. Microencapsulation of neonatal porcine islets: protection from human antibody/complement-mediated cytolysis in vitro and long-term reversal of diabetes in nude mice. Transplantation 2000; 69: 1084.

16. Murray AG, Nelson RC, Rayat GR, Elliott JF, Korbutt GS. - Neonatal porcine islet cells induce human CD4+, but not CD8+, lymphocyte proliferation and resist cell-mediated cytolytic injury in vitro. - Diabetes 1999 Sep;48(9):1713-9. 1713.

17. Korsgren O, Jansson L. - Characterization of mixed syngeneic-allogeneic and syngeneic-xenogeneic islet-graft rejections in mice. Evidence of functional impairment of the remaining syngeneic islets in xenograft rejections. - J Clin Invest 1994 Mar;93(3):1113-9. 1113.

18. Southern C, Schulster D, Green IC. - Inhibition of insulin secretion by interleukin-1 beta and tumour necrosis factor-alpha via an L-arginine-dependent nitric oxide generating mechanism. - FEBS Lett 1990 Dec 10;276(1-2):42-4. 42.

19. Scarim AL, Heitmeier MR, Corbett JA. - Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1beta. - Endocrinology 1997 Dec;138(12):5301-7. 5301.

20. Sandler S, Andersson A, Hellerstrom C. - Inhibitory effects of interleukin 1 on insulin secretion, insulin biosynthesis, and oxidative metabolism of isolated rat pancreatic islets. - Endocrinology 1987 Oct;121(4):1424-31. 1424.

21. Young AA, Gedulin BR, Bhavsar S, et al. - Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). - Diabetes 1999 May;48(5):1026-34. 1026.

22. Drucker DJ. - Glucagon-like peptides. - Diabetes 1998 Feb;47(2):159-69. 159.